AFAMRL-TR-83-62

(12)


# COMPARATIVE BIOCHEMISTRY AND METABOLISM: PART II: NAPHTHALENE LUNG TOXICITY

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AUGUST 1983



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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

Roger C. Inman

ROGER C. INMAN, Colonel, USAF
Chief
Toxic Hazards Division
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| REPORT DOCUMENTATION PAGE | | READ INSTRUCTIONS BEFORE COMPLETING FORM |
|---|---|---|
| 1. REPORT NUMBER<br>AFAMRL-TR-83-62 | 2. GOVT ACCESSION NO.<br>AD-A132943 | 3. RECIPIENT'S CATALOG NUMBER |
| 4. TITLE (and Subtitle)<br>COMPARATIVE BIOCHEMISTRY AND METABOLISM: PART II: NAPHTHALENE LUNG TOXICITY | | 5. TYPE OF REPORT & PERIOD COVERED<br>July 1982 - June 1983<br>6. PERFORMING ORG. REPORT NUMBER |
| 7. AUTHOR(s)<br>Alan R. Buckpitt, Ph.D. | | 8. CONTRACT OR GRANT NUMBER(s)<br>F33615-80-C-0512 |
| 9. PERFORMING ORGANIZATION NAME AND ADDRESS<br>The Regents of the University of California<br>University of California, Irvine<br>Irvine, CA 92717 | | 10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS<br>62202F, 6302, 01, 15 |
| 11. CONTROLLING OFFICE NAME AND ADDRESS<br>AFAMRL, Toxic Hazards Division<br>AMD, AFSC<br>Wright Patterson AFB, OH 45433 | | 12. REPORT DATE<br>August 1983<br>13. NUMBER OF PAGES<br>42 |
| 14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office) | | 15. SECURITY CLASS. (of this report)<br>Unclassified<br>15a. DECLASSIFICATION/DOWNGRADING SCHEDULE |

16. DISTRIBUTION STATEMENT (of this Report)

APPROVED FOR PUBLIC RELEASE: DISTRIBUTION UNLIMITED

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

AFAMRL Monitor: T.K. Pinkerton, AFAMRL/THT
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19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

naphthalene
lung toxicity
glutathione conjugates
reactive metabolites

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Previous studies have shown that intraperitoneal administration of naphthalene to mice results in dose-dependent necrosis of pulmonary bronchiolar epithelial cells but not in hepatic or renal damage. Several factors have supported a relationship between the cytochrome P-450 mediated formation of reactive naphthalene metabolites and the bronchiolar epithelial damage. Reactive metabolites from naphthalene become bound covalently to macromolecules in the lung in a dose and time dependent fashion and pretreatments which alter the severity of bronchiolar damage alter the pulmonary covalent binding in a parallel fashion.

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However, the observed target organ specificity for naphthalene-induced cytotoxicity is not reflected in the preferential covalent binding of reactive metabolites in the target tissue in vivo nor is it reflected in substantially higher rates of covalent binding in microsomes from target vs nontarget tissue. Thus, the purpose of the studies outlined in this annual report was to develop a method for examining the chemical nature of reactive metabolites produced in microsomes from target and nontarget tissues and to determine whether differences in the kinetics of formation of a particular reactive metabolite correlate with the highly species and organ specific toxicity of naphthalene. Electrophilic metabolites of naphthalene produced in microsomal incubation have been trapped, separated, and quantitated by HPLC. Since preliminary studies indicated that there were marked differences in the glutathione adduct profile in extracts from target and nontarget microsomal incubations and in extracts from lung microsomes of the hamster, a less sensitive species, additional studies were done to more fully characterize the microsomal metabolism of naphthalene to the dihydrodiol and to glutathione conjugates.

The in vitro formation of polar naphthalene metabolites was linear with time and microsomal protein, and the relative proportions of each of the metabolites remained relatively stable over the range of time and protein concentrations studied. The rate of naphthalene glutathione adduct formation increased markedly by addition of increasing amounts of cytosolic enzymes containing the glutathione transferases while the rate of dihydrodiol formation remained relatively constant. Addition of increasing quantities of partially purified glutathione transferases from phenobarbital-induced mouse liver resulted in a further increase in the rate of naphthalene glutathione adduct formation. Under incubation conditions where the cytochrome P-450 dependent formation of reactive naphthalene metabolites is the rate limiting step in the formation of glutathione adducts, total adduct formation in lung microsomal incubations was 150% that in liver microsomal incubations. Moreover, under these conditions the rate of conjugate 2 formation in lung microsomal incubations is more than 3 times that in liver microsomal incubations. The addition of lung cytosol to liver microsomal incubations slowed the overall rate of conjugate formation but the relative proportions of each of the conjugates remained constant. Likewise, addition of liver cytosol to lung microsomal incubations increased the overall rate of conjugate formation but did not alter the preferential formation of conjugate 2 relative to conjugates 1 and 3. Induction of microsomal epoxide hydrolases of naphthalene dihydrodiol and conjugate 2 failed to alter the rate of conjugate 1 or 3 formation. Inhibition of microsomal peroxide hydrolase by cyclohexene oxide decreased dihydrodiol formation, did not affect conjugate 1 and 3 formation and increased conjugate 2 formation. Addition of piperonyl butoxide or SKF 525A to hepatic microsomal incubations markedly decreased covalent binding of reactive naphthalene metabolites but only slightly decreased the rate of glutathione adduct formation. Dihydrodiol formation was increased by both inhibitors. Phenobarbital or 3-methylcholanthrene pretreatment produced a marked increase in the pulmonary microsome catalyzed formation of all four polar naphthalene metabolites. In comparison, phenobarbital increased the rates of formation of the dihydrodiol, conjugates 1 and 2 but not conjugate 3 in hepatic microsomes. 3-Methylcholanthrene, on the other hand, increased the rate of formation of the dihydrodiol and conjugate 2 but not conjugates 1 and 3. These studies suggest that metabolic pathways leading to covalent binding, dihydrodiol and glutathione adducts, may not arise from the same intermediate. In addition, it appears that the predominant formation of conjugate 2 in lung microsomal incubations in comparison to liver microsomal incubations is due to the regio- to stereospecificity of metabolism by cytochrome P-450 monooxygenase or epoxide hydrolase but not by the glutathione transferases.

## PREFACE

This is the annual report of the Subprogram on Comparative Biochemistry and Metabolism, Part II: Naphthalene Lung Toxicity and concerns work performed by the Department of Community and Environmental Medicine of the University of California, Irvine, on behalf of the Air Force under Contract #F33615-80-C-0512, Work Unit 63020115 This document describes the accomplishments of the subprogram from June 1982 through May 1983.

A.R. Buckpitt served as coordinator of Part II of the subprogram. Acknowledgment is made to Dr. Ronald Shank for his advice and encouragement in the DNA binding studies, to William Bosan and Eric Hunt for their advice and help in the analysis of binding to DNA, and to Linda Bahnson for her significant contributions to the research program.

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## INTRODUCTION

This report summarizes the work conducted from June 1982 through May 1983 in the subprogram on Comparative Biochemistry and Metabolism, Part II: Naphthalene Lung Toxicity. The mission of this subprogram is to provide further understanding of the relevant biochemical/metabolic events associated with the selective pulmonary bronchiolar epithelial cell necrosis observed in naphthalene-treated mice. The rationale for these studies and the importance to the USAF/USN will be discussed briefly in the following paragraphs.

Naphthalene is an ubiquitous environmental contaminant which is found in cigarette smoke and as a contaminant in air and water. Yearly, more than 300,000 metric tons of naphthalene are used in industry, primarily as a feed stock in the synthesis of dye intermediates (Priority Toxic Pollutants, 1980). Naphthalene is a starting material in the synthesis of decalin and tetrahydronaphthalene and is present in relatively large amounts in a solvent for ferrocene, a fuel additive currently under consideration for use by the USAF/USN (MacEwen and Vernot, 1981). Administration of naphthalene either intraperitoneally or as a vapor results in severe bronchiolar epithelial cell necrosis in mice, which appears to depend upon metabolism of the parent compound by the cytochrome P-450 monooxygenases (Warren et al., 1982). Since the bioactivation of chemically inert environmental agents may play a role in lung diseases such as fibrosis, emphysema and cancer (Boyd, 1980; Brody et al., 1981), it is important to understand factors which might influence the balance between toxifying and detoxifying pathways.

In addition, the elucidation of the toxicologically important biochemical and metabolic events occurring in naphthalene-exposed animals combined with the development of appropriate in vitro techniques for studying these processes will allow the determination of the potential sensitivity of human lung tissue to aromatic hydrocarbons like naphthalene. The mouse is substantially more sensitive to bronchiolar or alveolar cell damage induced by carbon tetrachloride (Boyd et al., 1980), 3-methylfuran (Boyd et al., 1978), butylated hydroxytoluene (Adamson et al., 1977), bromobenzene (Reid et al., 1973), and naphthalene (Buckpitt, 1981) than is the rat. Yet sufficiently detailed studies on the mechanism for the lung damage by these agents which would allow the appropriate studies to be conducted in human lung tissue have not been reported. Indeed, with few exceptions, studies on the metabolism of chemicals by human lung tissue have focused on benzo(a)pyrene (Prough et al., 1977; Sipal et al., 1979; McManus et al., 1980; Oesch et al., 1980). The ability of human lung tissue to metabolize those chemicals known to damage rodent lungs via the formation of toxic metabolites has not been explored. Since the mouse is used extensively in both short and long term toxicity testing, it is important to determine whether this species is an appropriate animal model for the human.

## BACKGROUND

The pulmonary bronchiolar necrosis resulting from intraperitoneal administration of naphthalene was first reported by Reid et al. (1973). Subsequent light and electron microscopic examination of the lungs of naphthalene-treated mice revealed that the nonciliated bronchiolar epithelial cell (Clara cell) was the primary target cell for naphthalene-induced damage and that the lung lesion was both time and dose-dependent (Mahvi et al., 1977). The finding that the Clara cell is a major locus of pulmonary cytochrome P-450 monooxygenases (Philpot and Wolf, 1981) and that naphthalene is metabolized to a number of reactive and potentially toxic epoxide and diol epoxide derivatives (Stillwell et al., 1981) suggested that naphthalene-induced bronchiolar damage may not be due to the parent compound but to the cytochrome P-450 dependent formation of toxic metabolites. Our initial

studies which have been described in detail (Buckpitt, 1981; Warren et al., 1982) indicate that metabolism of naphthalene by the cytochrome P-450 monooxygenase results in the formation of highly reactive metabolites which bind covalently to tissue macromolecules in vivo and which appear to conjugate with glutathione. Significant covalent binding and bronchiolar damage does not occur until doses of naphthalene are administered that are sufficient to deplete tissue glutathione substantially. Pretreatment with piperonyl butoxide blocks naphthalene-induced bronchiolar damage, glutathione depletion and covalent binding of reactive naphthalene metabolites. Likewise, prior depletion of tissue glutathione by diethyl maleate markedly increases covalent binding and pulmonary damage. While these studies supported a relationship between the formation and covalent binding of reactive metabolites and the bronchiolar damage observed after naphthalene, the pattern of organ toxicity did not coincide with the target organ for covalent binding of reactive metabolites (that is, high levels of binding occurred in lung, liver and kidney, but tissue damage was observed only in lung).

There are several possible explanations for this which are still consistent with a role of reactive metabolite formation in tissue damage. Metabolism of naphthalene may result in the formation of several different reactive species (1,2-oxide, 1,2,3,4-diepoxide or diol epoxide metabolites) all of which may be capable of binding irreversibly to tissue macromolecules but which may differ in their ability to interact with macromolecules critical to the survival of the cell. Thus, the nature of reactive metabolites produced could determine the organ specificity for damage. Other possibilities are that the macromolecules to which reactive naphthalene metabolites bind differ in target and nontarget tissue or that binding is highly localized in the lung but not in the liver or kidney. In addition, other data suggested that reactive metabolites of naphthalene which can become bound covalently to tissue macromolecules may be sufficiently stable to circulate, thereby indicating that measurements of covalent binding of radioactivity from $^{14}$C-naphthalene in vivo may not provide a reliable, quantitative indication of the formation of reactive, potentially toxic metabolites in situ (Buckpitt and Warren, 1983). Thus, the major emphasis of the studies conducted during the past contract year was to develop the methodology for examining the nature and rates of formation of reactive naphthalene metabolites in vitro and to characterize the metabolism of naphthalene to these reactive intermediates in target and nontarget tissue microsomes.

## RESEARCH PROGRAM

### Development of New Methodology for the Separation of Naphthalene Glutathione Adducts

Although previous studies had demonstrated that at least three glutathione adducts were found in hepatic microsomal incubations containing NADPH generating system, glutathione and cytosolic enzymes, the high pressure liquid chromatographic column/solvent systems being used for this work was unable to separate these adducts completely. Thus, the initial thrust of the studies conducted under 1982-1983 contract support was to test a variety of chromatographic conditions in an attempt to achieve complete separation of the highly polar naphthalene metabolites.

### General Methods

#### Animals

Male Swiss Webster mice weighing 30 to 30 g were purchased from Charles River Breeding Laboratories, Wilmington, MA for use in these studies. Hamsters were male, Golden Syrian (80 to 90 g) and were also purchased from Charles River. All animals were maintained in a HEPA/carbon filtered laminar flow cage rack and were given food and water ad libitum.

## Radiochemicals

$^{14}$C-Naphthalene (5 mCi/mmole), was purchased from Amersham Searle Corporation, Arlington Heights, IL and found to be > 99.8% radiochemically pure by high pressure liquid chromatography on a $C_{18}$ column. Stock solutions were diluted with unlabelled naphthalene to achieve specific activities of 600 to 1500 dpm/nmole as specified. L-Glutathione (glycine-2-[$^3$H]) (reduced form; 240 mCi/mmole) was purchased from New England Nuclear Corporation, Boston, MA and was used without further purification.

## Chemicals

Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP and butylated hydroxyanisole were purchased from Sigma Chemical Company, St. Louis, MO. Glutathione (reduced form) was from Calbiochem, La Jolla, CA. Cyclohexene oxide was from Aldrich Chemical Company, Milwaukee, WI. Piperonyl butoxide was purchased from Chemical Dynamics Corporation, South Plainfield, NJ and SKF 525A was a generous gift from the Smith Kline Corporation, Philadelphia, PA. All other chemicals were reagent grade or better.

## Preparation of Microsomal and Cytosolic Enzymes

Lung, liver and kidney microsomes were prepared by ultracentrifugation according to published procedures (Buckpitt and Boyd, 1980). The 100,000 g supernatant fraction (containing the glutathione transferases) from the first high speed centrifugation was chromatographed on a Sephadex G-25 column to remove endogenous glutathione. Microsomal and cytosolic protein concentrations were determined by the method of Lowry et al. (1951) or by the method of Bradford (1976).

## Incubations

Incubations were prepared on ice in a total volume of 2 ml, and contained microsomes (1-6 mg as specified), naphthalene (at the specified concentration added in 10 µl methanol), glutathione (concentrations as specified), an NADPH-generating system (cofactor) (Buckpitt and Boyd, 1980), and cytosolic enzymes (1-2 mg). The incubation vessels were capped and transferred to a shaking incubator at 37° C for the specified time. The vessels were again transferred to an ice bath, 4 ml of ice-cold methanol was added to stop the reaction, and the contents were transferred to centrifuge tubes.

## Covalent Binding

The covalent binding of radiolabel to precipitated microsomal protein was assayed as described previously (Buckpitt and Warren, 1983).

## Extraction and HPLC Analysis

After removing the protein by centrifugation, the methanol/water supernatant fraction was extracted twice with 3 ml portions of trimethylpentane to remove unmetabolized naphthalene. An aliquot (1-4 ml) of the methanol/water phase was evaporated to dryness under vacuum and was reconstituted in mobile phase (200-500 µl) for analysis. HPLC was done on a Waters ALC 201 liquid chromatograph with a Beckman 5 µm ODS column (0.46 x 25 cm or 0.46 x 15 cm) eluted with 5% acetontrile/1% glacial acetic acid/91-95% water at 1 ml/min. Radioactivity was collected into scintillation vials, 5-10 ml Beckman Econophase

was added, and the samples were counted for 20 min each in a Beckman 3150T liquid scintillation counter.

## RESULTS

Addition of glutathione to incubations of phenobarbital-induced (0.1% phenobarbital in the drinking water for 5 days) hepatic microsomes, [$^{14}$C]-naphthalene, cytosolic enzymes and an NADPH-generating system resulted in a marked decrease in the covalent binding of reactive naphthalene metabolites compared to incubations done in the absence of glutathione (Table 1). Addition of glutathione to complete incubations also resulted in the formation of three UV absorbing and radioactive peaks (eluting at 47, 53 and 58 min, Figure 1) which were not present in identical incubations without glutathione (Figure 1) or NADPH (data not shown). These same UV and radioactive peaks were present in extracts prepared from microsomal incubations containing naphthalene, cytosolic enzymes, NADPH-generating system and [$^{3}$H]-glutathione but were not present in incubations without cofactor (Figure 1) or without naphthalene (data not shown).

Table 1

Effect of Addition of Reduced Glutathione on the Covalent Binding of Reactive Naphthalene Metabolites to Hepatic Microsomal Protein*

| Incubation Conditions | | Covalent Binding† |
|---|---|---|
| Cofactor | Glutathione | |
| + | - | 21.1 ± 0.8 |
| + | + | 7.6 ± 1.6 |
| - | - | 0.1 ± 0.0 |

*Incubations were 20 min at 37° C and contained [$^{14}$C]-naphthalene (1 mM, 832 dpm/nmole), phenobarbital-induced microsomes (6 mg), cytosolic enzymes (2 mg), and glutathione (5 mM) or cofactor as specified.

†Values are the means ± S.E. of three incubations and are expressed as nmoles bound/mg protein/20 min.

### Identification of Polar Naphthalene Metabolites

The fact that the formation of the peak eluting at 41 min was dependent upon the presence of NADPH but not glutathione and was highly polar suggested that this derivative might be 1,2-dihydroxy-1,2-dihydronaphthalene. To test this possibility, naphthalene dihydrodiol was synthesized by reduction of 1,2-naphthoquinone (1 g) with $LiAlH_4$ (500 mg) for 6 hours at reflux. The pale brown product (165 mg) isolated from the reaction had a melting point of 103° C (reported 103° C) and an Rf of 0.25 on silica gel G in benzene/ethyl acetate/chloroform (1:1:1). This Rf, relative to that for naphthalene and 1-naphthol, was consistent with that previously reported for the dihydrodiol (Bock et al., 1976). Peak 1 (unidentified polar metabolite) had a retention time and UV spectra ($\lambda$max 260 nm) identical to that of synthetic naphthalene dihydrodiol.

Definitive identification of the naphthalene glutathione adducts is currently being done in collaboration with Dr. Neal Castagnoli, Professor of Chemistry and Pharmaceutical Chemistry, University of California, San Francisco. The approach to be used will include 500 mHz proton NMR and fast atom bombardment mass spectrometry.

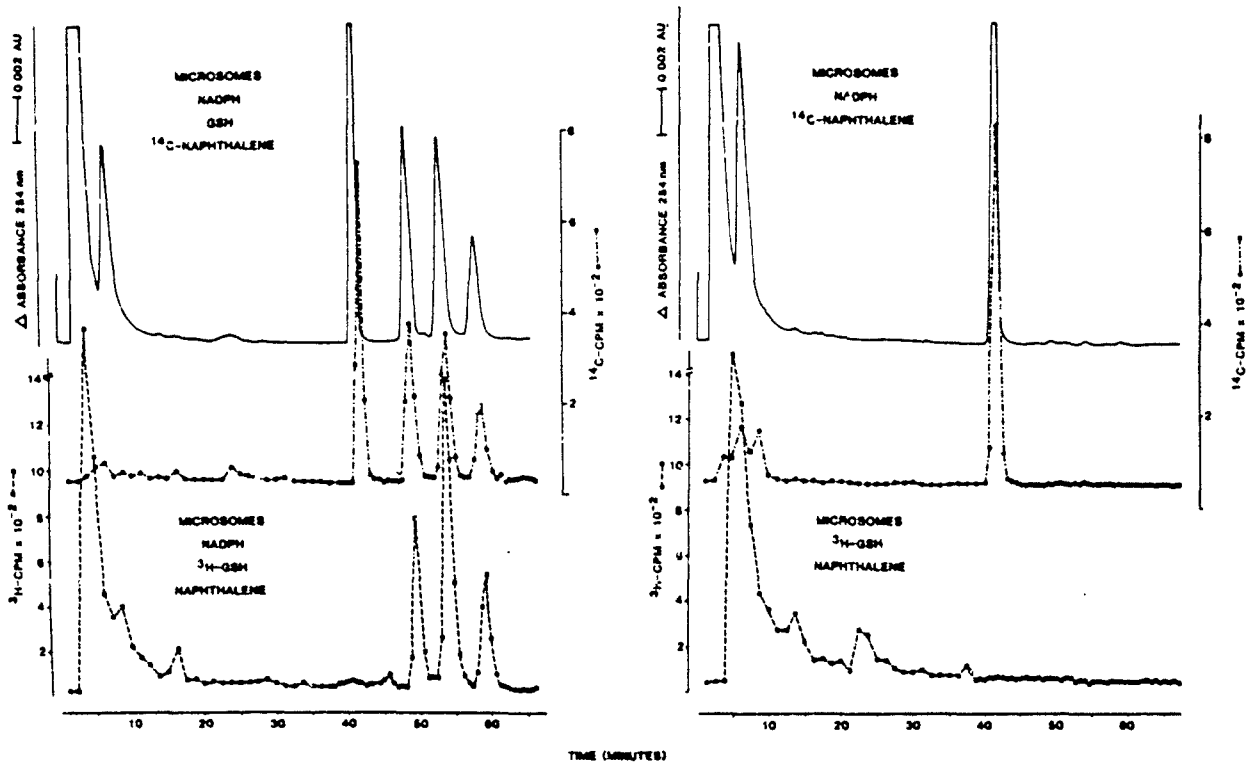


Figure 1. Ultraviolet (254 nm) and radioactive profiles of extracts from 20 min incubations of phenobarbital-induced liver microsomes (6 mg) with naphthalene and/or glutathione done under the following conditions: [$^{14}$C]-naphthalene (832 dpm/nmole, 1 mM), cytosolic enzymes (2 mg) and cofactor done in the presence or absence of 5 mM glutathione, or [$^{3}$H]-glutathione (1950 dpm/nmole, 0.1 mM), cytosolic enzymes and naphthalene (1 mM) done in the presence or absence of cofactor. Column eluant was collected at 1 min intervals for the first 15 min and at 30 sec intervals thereafter for scintillation counting.

Approximately 1 mg of conjugate 1 and conjugate 2 have been purified from a 300 ml incubation of liver microsomes (phenobarbital-induced mice), cofactor, glutathione and cytosolic enzymes incubated for 30 min at 37°C. One volume of ice cold methanol was added to stop the reaction and the precipitated protein was removed by centrifugation at 18,000 x g for 20 min. The supernatant was evaporated under vacuum, reconstituted in 20 ml water and extracted with trimethylpentane (2 × 200 ml). The remaining aqueous phase was chromatographed in 5 ml increments on a column (10 × 1 cm) packed with Amberlite XAD-2 resin. The column was washed with 100 ml 1% acetic acid/99% water followed by 100 ml methanol to elute the naphthalene glutathione adducts. These were then separated on a Beckman Ultrasphere (5 μ, $C_{18}$, 1 × 25 cm) column eluted with 6-8% acetonitrile/1% acetic acid/water at 3 ml/min. The glutathione adducts were collected, evaporated in round bottom flasks and reconstituted in 2 ml water. Five hundred microliter aliquots of this were rechromatographed on the Beckman Ultrasphere semipreparative column and the conjugates were collected. An aliquot of each of the purified conjugates was rechromatographed on a Waters Nova Pak column (0.8 × 10 cm, 4 μ spherical packing) using 5% acetonitrile/1% acetic acid/94% water at 2 ml/min to assess the purity of each conjugate. Glutathione adduct 1 and 2 chromatographed as a single UV absorbing peak and each was judged to be at least 99% pure based on the UV chromatographic tracing. The majority of glutathione adduct 3 was lost during the final chromatography step and thus less than 1 μ is available for analysis. All three samples are currently in Dr. Castagnoli's laboratory awaiting analysis.

**Rates of Conjugate Formation in Microsomes from Target vs Non-Target Tissues of the Mouse and in Lungs of Sensitive vs Non-Sensitive Species**

To provide a preliminary indication of whether the relative rates or nature of glutathione adducts formed in target vs nontarget tissues of the mouse differed, $^{14}$C-naphthalene was incubated with lung, liver or kidney microsomes in the presence of glutathione, NADPH generating system, and cytosolic enzymes. As shown in Figure 2 the radioactive profiles of extracts prepared from lung, liver or kidney microsomal incubations differed markedly. Lung microsomes preferentially form naphthalene glutathione adduct 2 (10-20:1) while the ratio of conjugate 2/1 and 2/3 in liver microsomes does not exceed 2:1. Kidney, a non-target tissue of the mouse, metabolizes naphthalene to naphthalene dihydrodiol and to glutathione adducts very slowly.

Previous studies have shown that ip administration of naphthalene to hamsters results in bronchiolar epithelial cell necrosis but only at doses which are 3 to 5 times higher than a lung toxic dose in the mouse. To explore the possibility that the relative rates of formation of glutathione adducts correlate with the relative tissue and species sensitivity to the cytotoxic effects of naphthalene, naphthalene glutathione adduct formation has been measured in hamster lung microsomal incubations. The data in Table 2, comparing the rates of formation of naphthalene dihydrodiol and conjugates in mouse and hamster, indicate that the formation of the dihydrodiol and conjugates 1 and 3 occur at approximately the same rate in mouse and hamster lung microsomal incubations. In contrast, hamster lung microsome-catalyzed formation of conjugate 2 occurs at a rate which is less than 1% the rate of formation of conjugate 2 in mouse lung microsomal incubations. These observations provide additional support for the possibility that the rate of formation of a particular reactive metabolite from naphthalene (which is trapped as conjugate 2) plays a critical role in determining the target organ for toxicity by naphthalene. This hypothesis will require considerably more experimental support, and the studies planned in the remaining 3 months of the contract year will continue to test this hypothesis.

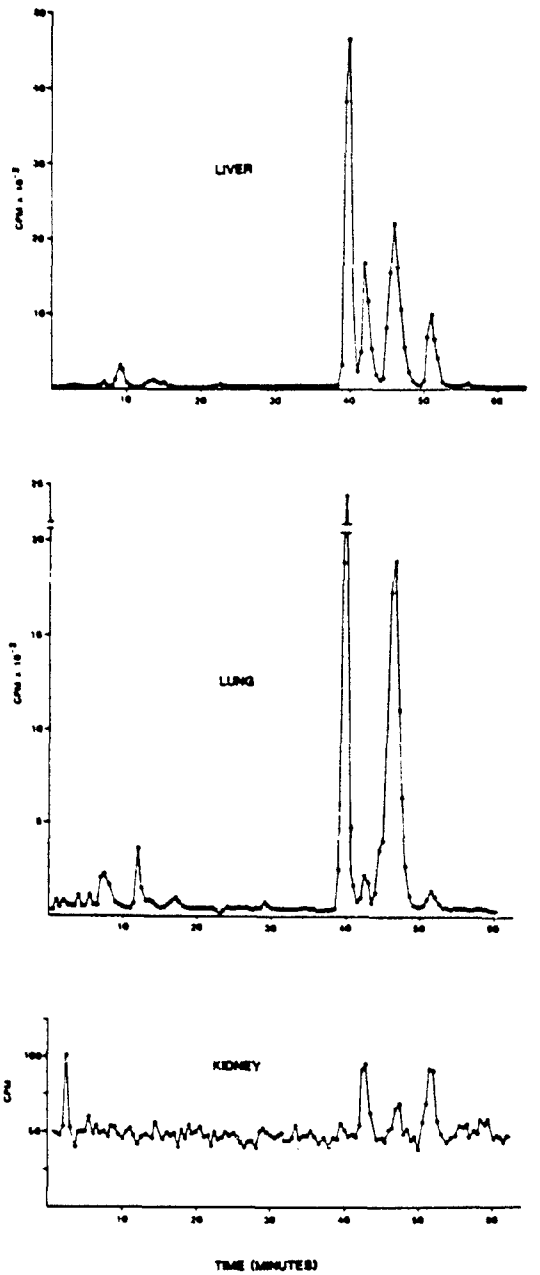


Figure 2. Radioactive profiles of extracts from uninduced mouse liver, lung or kidney microsomal (2.5 mg) incubations with [$^{14}$C]naphthalene (1 mM, 1247 dpm/nmole), cofactor, cytosolic enzymes (1 mg) and glutathione (5 mM). Incubations were for 15 min at 37°C; extracts were prepared in an identical fashion, and radioactivity was collected from the column at 30-sec intervals.

Table 2

Rates of Formation of Naphthalene Dihydrodiol and of Naphthalene-Glutathione Adducts in Mouse and Hamster Lung Microsomal Incubations

| | nmoles/min/mg Protein | | | |
|---|---|---|---|---|
| Species | Naphthalene Dihydrodiol | Conj 1 | Conj 2 | Conj 3 |
| Mouse* | 1.26 ± 0.04 | 0.16 ± 0.01 | 4.10 ± 0.13 | 0.15 ± 0.01 |
| Hamster† | 1.62 | 0.16 | 0.02 | 0.27 |

*Incubations contained lung microsomes (2 mg), $^{14}$C-naphthalene (1280 dpm/nmole), glutathione (5 mM), lung cytosol (2 mg), and NADPH generating system. Incubations were for 6 min at 37° C. Values are the mean ± S.E. for three incubations.

† Incubation conditions were identical to those in * except hamster lung microsomes were used and the incubation was for 7.5 min. Values are the mean of 2 incubations.

**Studies of the Kinetics of Formation of Polar Naphthalene Metabolites**

Examination of the kinetics of cytochrome P-450 dependent formation of reactive naphthalene metabolites trapped as glutathione adducts will be difficult and must be interpreted with considerable caution. Not only are impure enzyme sytems being used (i.e. microsomes) but the formation of the glutathione adduct requires both the cytochrome P-450 dependent formation of the intermediate and the glutathione transferase-catalyzed formation of the conjugate. To monitor the kinetics of intermediate formation, incubation conditions must be chosen to promote complete conversion of intermediate to conjugate (i.e. intermediate formation must be rate limiting). Nevertheless, there is substantial evidence, illustrated by studies with the pulmonary toxicant 4-ipomeanol, to suggest that the kinetics of reactive intermediate formation may play a critical role in determining the target organ for damage. For example, at saturating substrate concentrations, metabolism of 4-ipomeanol to covalently bound products in rat, mouse and guinea pig, lung and liver microsomal incubations occurred at similar rates (Dutcher and Boyd, 1979) and thus did not reflect the relative sensitivity of lung vs liver for 4-ipomeanol-induced cytotoxicity. Yet, complete kinetic analysis indicates that the $K_m$ for the covalent binding reaction in rat lung microsomes is 10 fold lower than in liver microsomes (Boyd et al., 1978). Similarly, the $K_m$ for the covalent binding in rabbit lung microsomes is approximately half the $K_m$ in liver microsomes (Wolf et al., 1982). These kinetic data are consistent with the relative ratios of covalent binding in lung and liver of the rat and rabbit in vivo. It is important to remember that the concentration of a given cytotoxicant in the target cell in vivo is likely to be quite low, and, therefore, the $K_m$ for the formation of a cytotoxic metabolite may be critical to the expression of toxicity.

Time Course Formation of Polar Naphthalene Metabolites

Studies of the time dependency of formation of the polar naphthalene metabolites were done for two reasons: 1) to establish conditions appropriate for Michaelis Menton kinetics (see above); and 2) to determine whether the formation of any of the metabolites required recycling through the monooxygenase system. The data in Figure 3 indicate that the formation of each of the metabolites is linear for the entire 16 min incubation period (at least

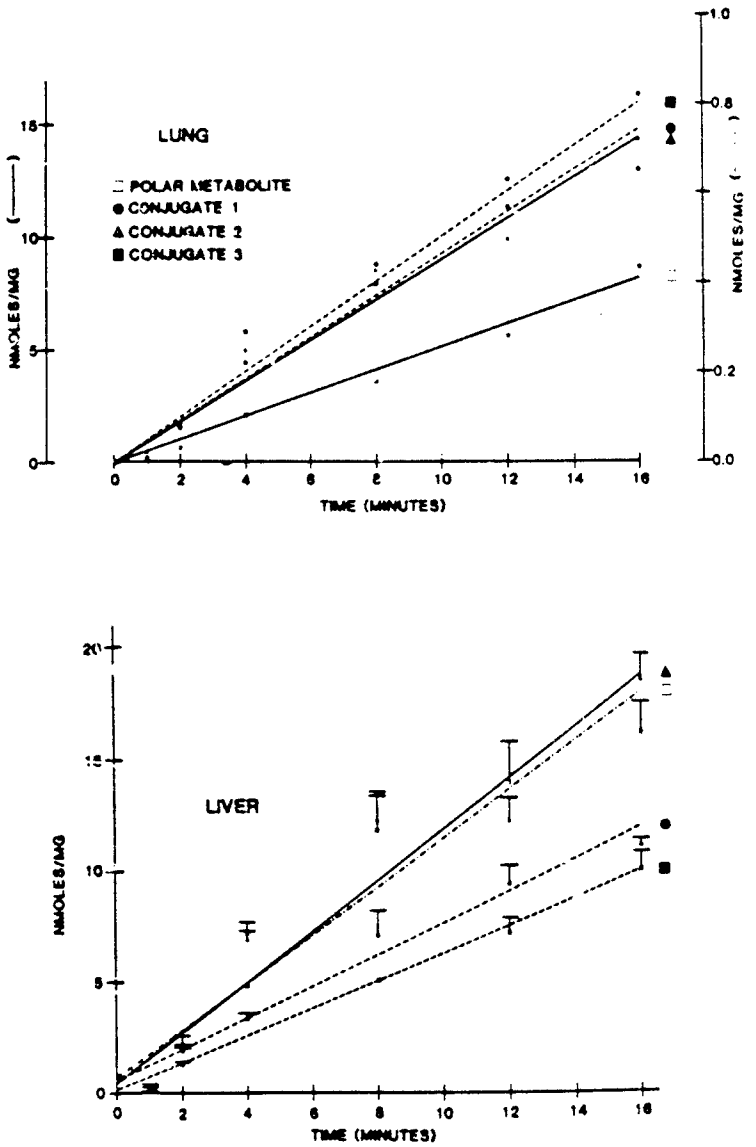


Figure 3. Time course formation of a polar metabolite ($R_t$ 41.0 min) and naphthalene glutathione adducts by mouse lung and liver microsomes. Incubations were prepared on ice and contained microsomes (2 mg), NADPH generating system, glutathione (5 mM), supernatant enzymes (2 mg) and $^{14}$C-naphthalene (1 mM, 1355 dpm/nmole). Incubation vessels were preincubated for 2 min at 37° C, substrate was added and the vessels were capped and incubated for the indicated time. Values are the mean of 2 (lung) or 3 (liver) incubations. Errors are S.E.M.

within the limits of measurement error). All subsequent incubations have been conducted for 6 min which is well within the linear portion of the time curve. Additionally, the data in Figure 3 show that there was no time lag in the formation of any of the metabolites nor was there a significant change in the ratios of metabolite over time. Thus, the formation of any of the three conjugates probably does not involve subsequent metabolism of one of the other conjugates (i.e. conjugate 2 does not arise through further metabolism of conjugate 1).

Linearity with Microsomal Protein

The data in Figure 4 indicate that the formation of the naphthalene dihydrodiol and the naphthalene glutathione adducts is linearly dependent upon the concentration of microsomal protein to approximately 4 mg (2 mg/ml) in both lung and liver. The relative proportion of conjugates forming during the microsomal metabolism of naphthalene remained nearly constant with increasing amounts of microsomal protein up to 4 mg.

Effect of Addition of Varying Amounts of Supernatant Enzymes

In addition to catalyzing the conjugation of a variety of inherently electrophilic compounds with reduced glutathione, the glutathione S-transferases have been shown to promote glutathione conjugation with microsomally-generated reactive metabolites of bromobenzene and acetaminophen (Monks et al., 1982; Rollins and Buckpitt, 1979). However, highly reactive metabolites from 4-ipomeanol formed glutathione conjugates without the aid of the cytosolic transferase enzymes (Buckpitt and Boyd, 1980), thus suggesting that the ability of the transferase enzymes in cytosol to stimulate glutathione conjugate formation depends upon the chemical reactivity of the substrate (see discussion by Ketterer, 1983). The ability of cytosolic transferases to catalyze glutathione conjugation with reactive metabolites also appears to correlate with whether a particular agent shows a glutathione dose threshold for covalent binding and tissue necrosis.

Thus, to explore the possibility that cytosolic glutathione transferases promote the conjugation of reactive naphthalene metabolites with glutathione and to establish conditions necessary for maximal rates of conjugate formation, varying amounts of lung and liver cytosolic enzymes were added to incubations containing microsomes, NADPH generating system, glutathione and $^{14}$C-naphthalene. The data in Figure 5 indicate that the rate of formation of all three glutathione adducts is enhanced substantially by the addition of increasing amounts of cytosolic enzymes. The addition of increasing quantities of lung cytosol had no significant effect on the rate of formation of polar metabolite 1. With liver enzyme, the formation of polar metabolite 1 in incubations containing 2 mg cytosolic protein occurred at a slightly decreased rate (87%) compared to incubations with 2 mg boiled enzyme. These studies provide further support for the view that there is an association between a glutathione dose threshold in vivo, the stability of reactive metabolites which become conjugated to glutathione and a role for cytosolic glutathione transferases in the formation of these conjugates. These studies also indicate that even with the addition of 2 mg cytosolic protein, not all of the reactive metabolites generated by the microsomal metabolism of naphthalene are trapped as glutathione adducts.

**Effect of Tissue Source on the Relative Rates of Naphthalene Glutathione Conjugate Formation**

Since it appeared that partially purified glutathione transferases would be required to make the cytochrome P-450 monooxygenase-mediated metabolism of naphthalene the rate limiting step in glutathione adduct formation and since these enzymes are most easily purified from liver, it was necessary to demonstrate that the preferential formation of glutathione

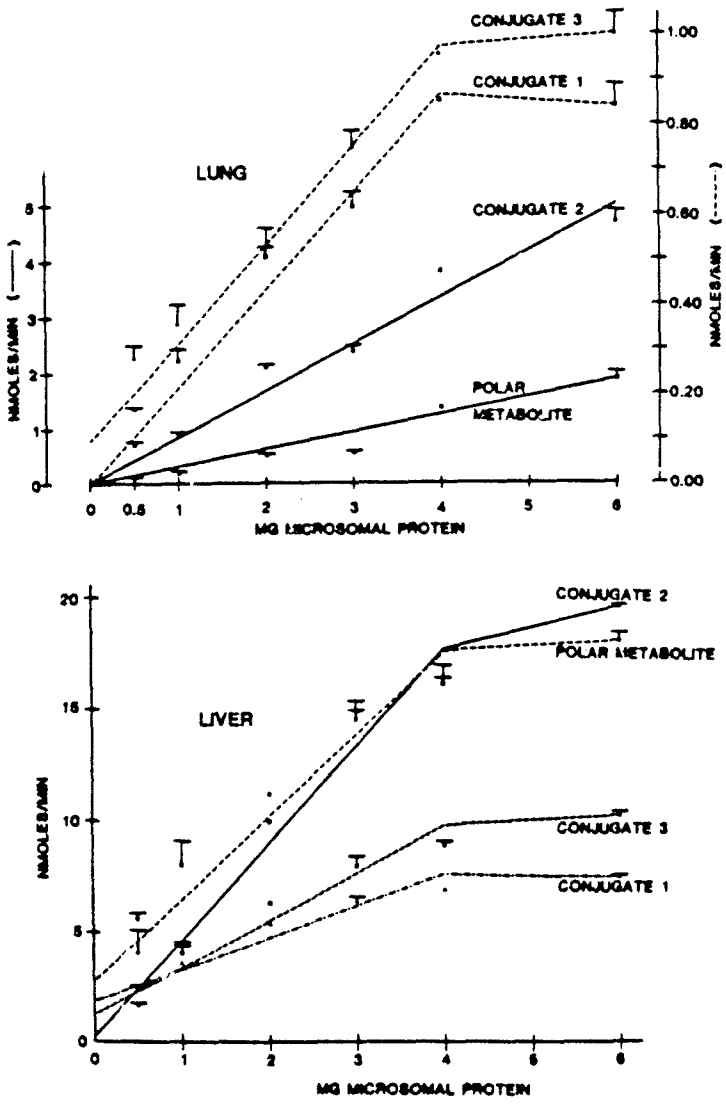


Figure 4. Dependency of rates of formation of a polar metabolite and of naphthalene-glutathione adducts on microsomal protein content. Incubations contained microsomes, NADPH generating system, glutathione (5 mM), supernatant enzymes (1 mg) and $^{14}$C-naphthalene (1 mM, 1075 dpm/ nmole). Incubations were for 6 min at 37°C. Values are the mean ± S.E. for three incubations.

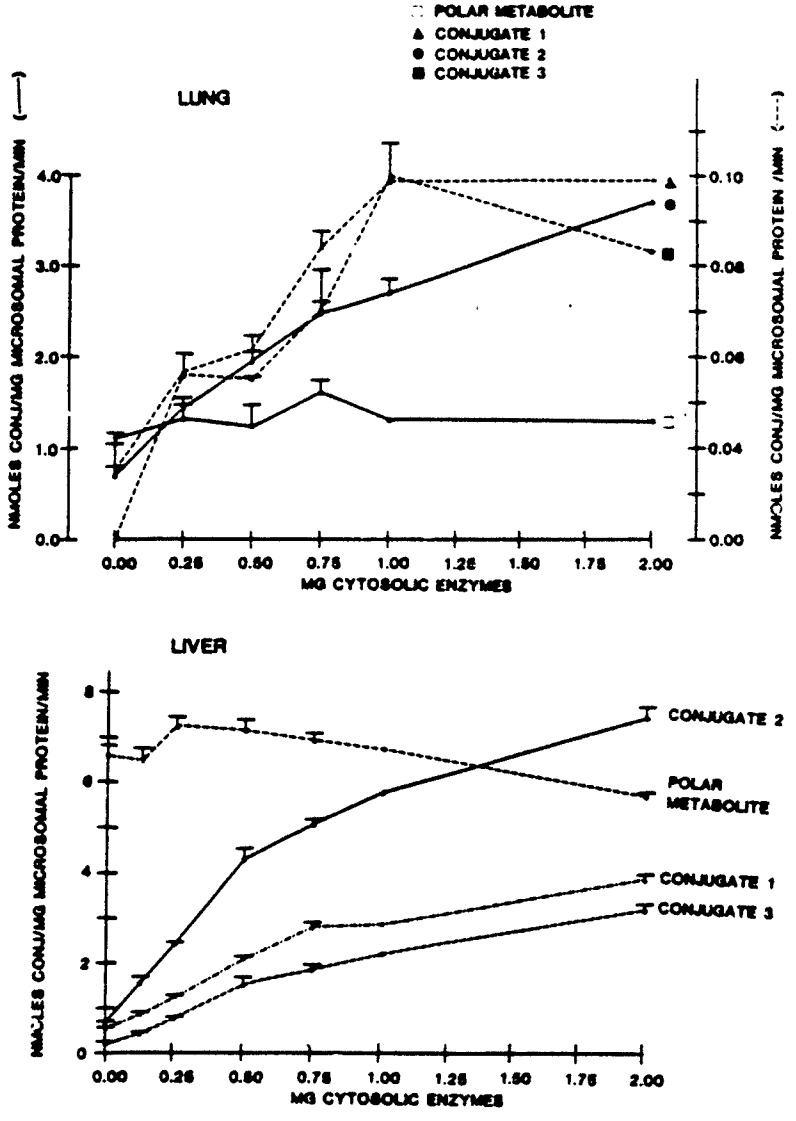


**Figure 5. Effect of addition of varying amounts of supernatant enzyme protein on the rates of formation of a polar metabolite and naphthalene-glutathione adducts. Incubations contained 2 mg microsomal protein, NADPH generating system, glutathione (5 mM), $^{14}$C-naphthalene (1 mM, 1317 dpm/nmole) and varying amounts of native cytosolic enzymes. The amount of cytosolic enzyme protein was brought to 2 mg by the addition of boiled cytosolic enzyme protein. Incubations were for 6 min at 37° C. Values are the mean ± S.E. for three incubations.**

adduct 2 in lung microsomal incubations was not due to the specificity of metabolism by cytosolic glutathione transferases.

Thus, the rates of conjugate formation were monitored in incubations of liver microsomes with lung cytosol and lung microsomes with liver cytosol. Consistent with the results of previous studies, the rate of formation of conjugate 2/1 or 2/3 was > 25/1 in lung microsomal/lung cytosol incubations (Tables 3 and 4). Substitution of liver cytosol in lung microsomal incubations nearly doubled the rate of formation of all three conjugates but altered the relative ratios of conjugates formed only slightly. Conversely, the addition of lung cytosol to liver microsomal incubations slowed the rates of formation of all of the conjugates but failed to substantially alter the relative ratios of conjugate formed. Therefore, it appears that the metabolic selectivity of the microsomal cytochrome P-450 monooxygenases rather than the cytosolic glutathione transferases are important in determining the relative rates of formation of each of the naphthalene glutathione adducts. In addition, the data in Table 3 showing that the rate of conjugate formation in lung microsomal incubations with liver cytosol is substantially higher than identical incubations done with lung cytosol further support the need for use of semipurified glutathione transferases in the kinetic studies on conjugate formation.

Table 3

Comparison of Naphthalene-Glutathione Adduct Formation in Lung and Liver Microsomes and Effect of Tissue Source of Cytosolic Enzymes*

| Incubation Conditions | | | | Polar Naphthalene Metabolites (nmoles/mg/min) | | | | Total GSH Conj |
|---|---|---|---|---|---|---|---|---|
| Microsomes | Cytosol | NADPH | GSH | Dihydrodiol 1 | Conj 1 | Conj 2 | Conj 3 | (nmoles/mg/min) |
| Lung | Lung | + | - | 1.19 ± 0.10 | 0.06 | 0.16 | 0.05 | — |
| Lung | Lung | + | + | 1.26 ± 0.04 | 0.16 ± 0.01 | 4.10 ± 0.13 | 0.15 ± 0.01 | 4.41 |
| Liver | Liver | + | - | 3.41 ± 0.08 | 0.10 | 0.11 | 0.08 | — |
| Liver | Liver | + | + | 2.21 ± 0.07 | 2.12 ± 0.09 | 3.68 ± 0.13 | 1.84 ± 0.06 | 7.84 |
| Lung | Liver | + | + | 1.06 ± 0.17 | 0.35 ± 0.03 | 7.08 ± 0.48 | 0.34 ± 0.06 | 7.77 |
| Liver | Lung | + | + | 2.52 ± 0.14 | 0.95 ± 0.06 | 2.10 ± 0.45 | 0.48 ± 0.03 | 3.53 |

*In a total volume of 2 ml, incubations contained $^{14}$C-naphthalene (1 mM, 1280 dpm/nmole), microsomes (2 mg cytosol (2 mg) and NADPH generating system and glutathione (5 mM) where indicated. Incubations were conducte for 6 min at 37° C. Values are the mean ± S.E. for triplicate incubations.

Table 4

Effect of Source of Cytosolic Enzymes on the Ratio of Conjugate 2/Conjugate 1 and Conjugate 2/Conjugate 3*

| Tissue Source | | Ratio of Rates of Conjugate Formation | |
|---|---|---|---|
| Microsomes | Cytosol | 2/1 | 2/3 |
| Lung | Lung | 26 | 27 |
| Lung | Liver | 20 | 21 |
| Liver | Liver | 2 | 2 |
| Liver | Lung | 2 | 6 |

*Data taken from Table 3.

Effect of Addition of Varying Amounts of Semipurified Glutathione Transferases

Glutathione S-transferases were partially purified by affinity chromatography (Simons and Vander Jagt) from 100,000 x g supernatant isolated from liver of phenobarbital-treated (0.1% phenobarbital in the drinking water for 5 days) mice. The cytosolic enzymes were chromatographed on Sephadex G-25 to remove reduced glutathione, and then 5 ml were applied to a 6.5× 1 cm column packed with glutathione agarose affinity media (Sigma #G-4510), previously equilibrated with 22 mM sodium phosphate buffer, pH 7.0. Under these conditions, the column retained greater than 90% of the applied glutathione transferase enzymes (activity assayed with 1-chloro-2,4-dinitrobenzene (CDNB) according to Habig et al., 1976). After rinsing the column with a further 7 ml 22 mM phosphate buffer, glutathione transferase activity was eluted with 0.05 M Tris pH 9.6 containing 5 mM glutathione. This fraction was concentrated by pressure dialysis using an Amicon PM 10 membrane, and the concentrated protein solution was washed with 4 volumes of 22 mM phosphate buffer. Sufficient glycerol, EDTA and glutathione were added to bring the solution to 10% glycerol, 0.1 mM EDTA and 2 mM glutathione. Activity of this fraction was found to be 16.5 μ moles CDNB conjugating activity/mg protein/min indicating that the affinity column purified enzyme was approximately three-fold purified from cytosol.

To determine whether sufficient quantities of the semipurified glutathione transferase could be added to microsomal incubations to make the initial cytochrome P-450 mediated metabolism of naphthalene the rate determining step in conjugate formation, mouse lung and liver microsomes were incubated with naphthalene, glutathione, cofactor and 1, 2, 4, 8, 16 or 32 CDNB units of glutathione transferase activity. Conjugate formation and covalent binding were assayed as described previously.

The data in Figure 6 show that the rate of glutathione adduct formation increased substantially over the range of 1 to 8 CDNB transferase units in both lung and liver microsomal incubations. Since 1 mg cytosol from control liver normally has glutathione transferase activities with CDNB in the range of 1 μ mole/min/mg protein, previous studies in which 2 mg cytosolic enzymes were used proceeded at about half the maximal rate. Addition of increasing amounts of transferase enzyme from 8 to 32 CDNB units slightly increased the rate of formation of conjugate 2 but did not affect the rates of formation of either conjugates 1 or 3. In contrast to the previous studies using 100,000 x g supernatant as a source of glutathione transferase activity, the studies with semipurified transferase indicated that the

rate of dihydrodiol formation decreased with the addition of increasing amounts of cytosolic enzyme. In previous studies using lung microsomes/lung cytosol, and liver microsomes/liver cytosol the rate of total conjugate formation in incubations prepared with hepatic enzymes were considerably higher than with lung. In contrast, the data in Figure 6 indicate that total conjugate formation in lung microsomal incubations is nearly double that in liver microsomes. In addition, conjugate 2 formation proceeds at a rate which is nearly three-fold higher in lung microsomes than in liver. The ratio of conjugate 2/1 and 2/3 is approximately 20:1 in lung microsomal incubations while it is 2:1 in liver microsomal incubations with purified transferase. Thus, although the addition of semipurified transferase markedly increased the rate of formation of all three glutathione adducts in lung and liver microsomes, it did not alter the relative rates of conjugate formation.

Duplicate incubations were prepared on ice in a total volume of 2 ml and contained: 1 mg microsomal protein, 1 mM $^{14}$C-naphthalene (1592 dpm/nmole), 5 mM glutathione, NADPH generating system, and 1, 2, 4, 8, 16 or 32 units of glutathione transferase activity (CDNB). Incubations were conducted for 6 min at 37° C.

The data in Table 5 indicate that the covalent binding of reactive metabolites from naphthalene at all concentrations of transferase studied is low in comparison to the quantity of metabolite trapped as glutathione adducts. For example, total conjugate formation in lung microsomal incubations with 8 CDNB units of glutathione transferase activity was approximately 14 nmoles/min/mg while covalent binding was 0.15 nmoles/min/mg. These data indicate that nearly all of the reactive metabolites can be trapped as glutathione adducts and that monitoring adduct formation can provide an accurate determination of the amount of naphthalene metabolized to unstable derivatives.

Table 5

Covalent Binding of Reactive Metabolites from Naphthalene In Vitro: Effect of Addition of Varying Amounts of Semipurified Glutathione Transferases*

| Glutathione Transferase CDNB units (μ moles/min) | Covalent Binding nmoles/mg/min* Lung | Liver |
|---|---|---|
| 1 | 0.23 | 0.37 |
| 2 | 0.22 | 0.35 |
| 4 | 0.19 | 0.30 |
| 8 | 0.15 | 0.24 |
| 16 | 0.10 | 0.13 |
| 32 | 0.08 | 0.06 |

*Values are the mean for 2 incubations.

**Factors Which Modify the Rates of Formation of Polar Naphthalene Metabolites**

Effect of Phenobarbital or 3-Methylcholanthrene Treatment

In contrast to control mice where administration of naphthalene does not result in hepatocellular necrosis, prior treatment with the cytochrome P-450 monooxygenase inducers phenobarbital or 3-methylcholanthrene resulted in centrilobular necrosis 24 hours after administration of 400 mg/kg naphthalene, ip. In addition, phenobarbital but not

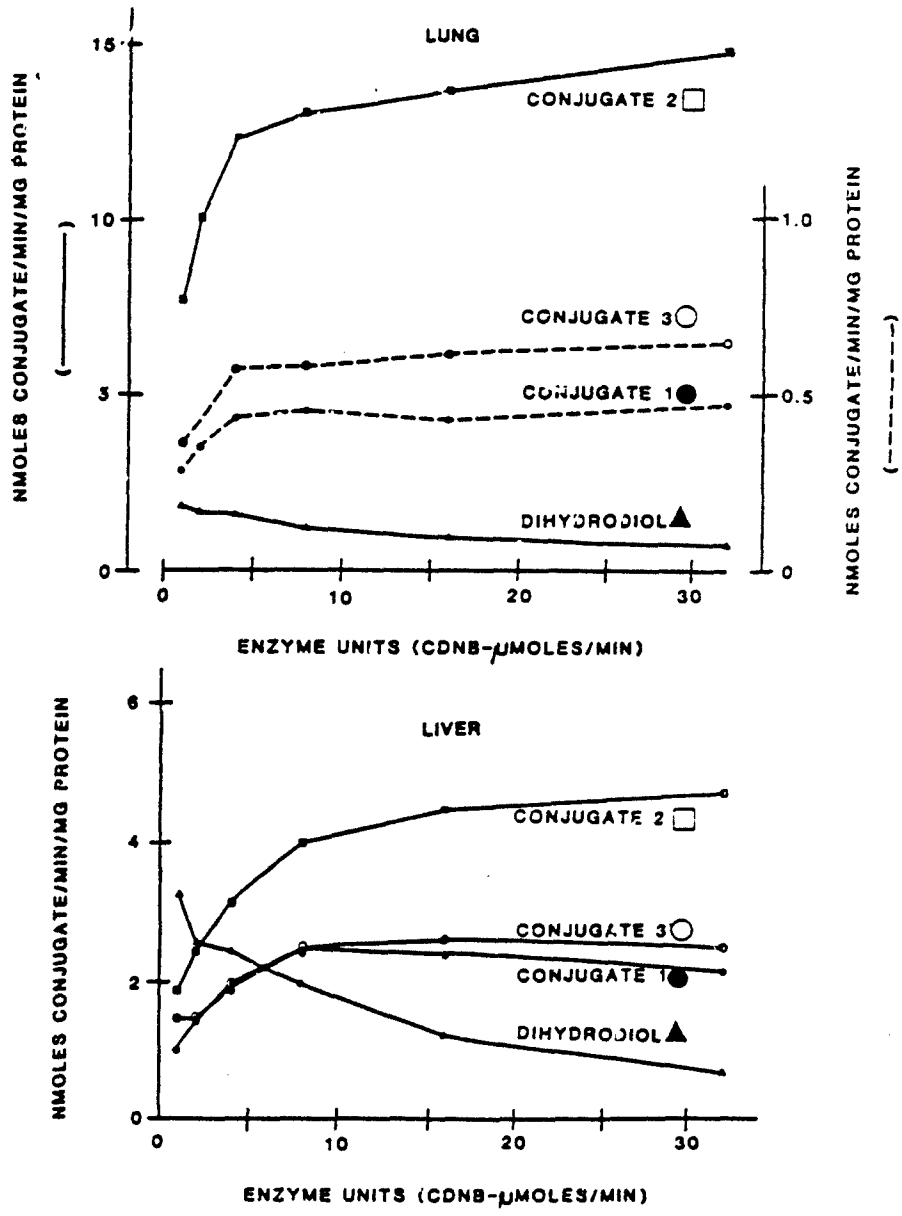


**Figure 6. Effect of addition of varying amounts of semipurified glutathione transferases on the rate of formation of glutathione adducts in mouse lung and liver microsomal incubations.**

3-methylcholanthrene pretreatment increased the covalent binding of reactive naphthalene metabolites in all tissues studied 4 hours after administration of $^{14}$C-naphthalene. Liver microsomes from phenobarbital but not 3-methylcholanthrene-induced animals metabolized naphthalene to covalently bound products at significantly higher rates than control while the rate of formation of reactive naphthalene metabolites in lung microsomes from phenobarbital and 3-methylcholanthrene pretreated animals was lower than control (Buckpitt and Warren, 1983). Since phenobarbital induction altered the sensitivity of the liver to naphthalene-induced cytotoxicity and since both phenobarbital and 3-methylcholanthrene markedly decreased the rate of covalent binding in lung microsomes, studies were done to determine whether cytochrome P-450 monooxygenase-induction by phenobarbital or 3-methycholanthrene would lead to an alteration of the ratio of glutathione conjugates formed.

The data in Figure 7 indicate that prior treatment with either phenobarbital or 3-methylcholanthrene resulted in substantially increased rates of formation of the naphthalene dihydrodiol (polar metabolite) and conjugate 1, 2, and 3 in lung microsomal incubations compared with control. However, neither pretreatment significantly altered the ratios of the conjugates formed. These data on the effects of phenobarbital induction are surprising for a number of reasons. While one of the isozymes of cytochrome P-450 isolated from rat and rabbit lung is similar (if not identical) to the major phenobarbital-inducible form of cytochrome P-450 in the liver, pulmonary cytochrome P-450 is unaffected by phenobarbital treatment (Philpot and Wolf, 1982). In addition, our previous studies indicated that phenobarbital induction resulted in decreased lung microsomal covalent binding of naphthalene metabolites. The substantial increase in pulmonary microsomal naphthalene metabolism resulting from 3-methylcholanthrene pretreatment is consistent with the inducibility of pulmonary P-450 by polycyclic aromatic hydrocarbons.

The increase in the rates of formation of the naphthalene dihydrodiol and glutathione adducts 1 and 2 by phenobarbital pretreatment are consistently less pronounced in liver microsomal incubations than in lung. Pretreatment with 3-methylcholanthrene appeared to have no effect on the rate of liver microsomal metabolism of naphthalene to any of the polar metabolites with the possible exception of conjugate 2. The preferential increase in formation of conjugate 2 by 3-methylcholanthrene induction indicates that the presence of different forms of the cytochrome P-450 isozymes in liver vs lung may be the basis for the marked differences in the ratios of conjugates formed by microsomes from these two tissues.

Effect of Induction or Inhibition of Epoxide Hydrolase on the Rates of Polar Naphthalene Metabolite Formation

Epoxide hydrolase-catalyzed formation of dihydrodiols represents a major pathway for arene oxide detoxification. Since metabolism via this pathway potentially competes with the glutathione conjugation pathway, it was important to determine whether induction or inhibition of microsomal epoxide hydrolase influenced not only the overall rate of glutathione conjugate formation but the relative ratios of conjugates formed as well.

Previous studies by Cha et al. (1978) have demonstrated that the addition of 0.75% butylated hydroxyanisole to the diet results in marked increases in microsomal epoxide hydrolase and cytosolic glutathione transferases but no change in cytochrome P-450 level. Therefore, liver microsomes were prepared from male Swiss Webster mice fed control or BHA treated powdered diet for 14 days prior to sacrifice. Cytochrome P-450 levels, measured by the method of Omura and Sato (1963), were not altered significantly by BHA treatment while styrene oxide epoxide hydrolase activity was increased three-fold (Figure 8). Induction of epoxide hydrolase activity resulted in a marked decrease in covalent binding of reactive naphthalene metabolites in incubations containing cofactor but no glutathione and in a marked increase in the rate of dihydrodiol formation in incubations containing either 0.5 or 5 mM

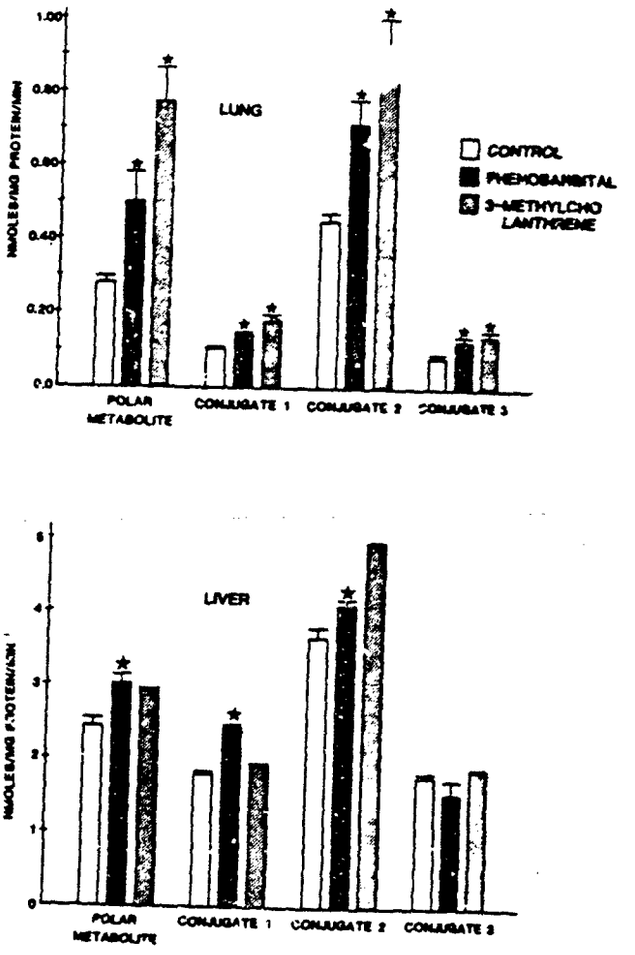


**Figure 7. Effect of pretreatment with phenobarbital or 3-methylcholanthrene on the pulmonary and hepatic microsomal metabolism of naphthalene to a polar metabolite and to glutathione adducts. Incubations contained 2 mg microsomal protein, NADPH generating system, glutathione (5 mM), cytosolic enzymes from control animals (1 mg), and $^{14}$C-naphthalene (1,111 dpm/nmole). Incubations were for 6 min at 37°C. Values are the mean ± S.E. of three incubations.**

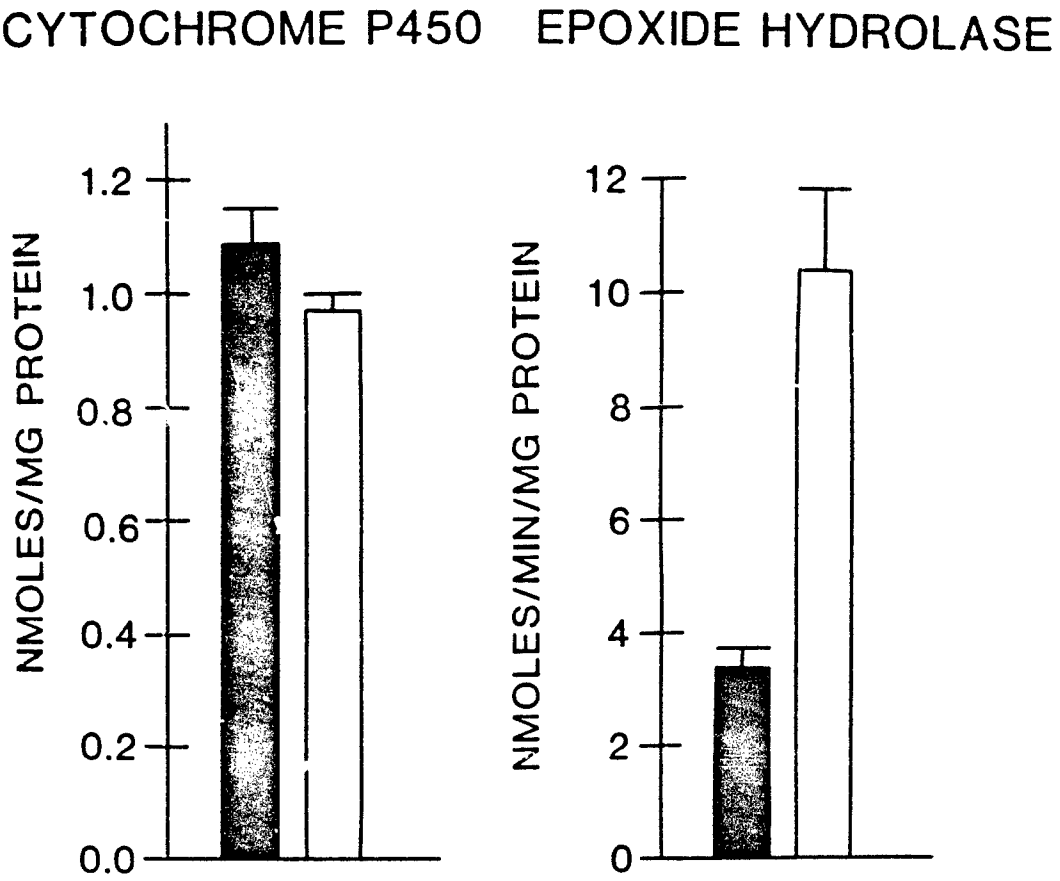


Figure 8. Effect of dietary BHA (0.75%) on hepatic microsomal cytochrome P-450 and on epoxide hydrolase. Liver microsomes were prepared from individual livers of 5 control (shaded bar) and 5 BHA treated mice, and cytochrome P-450 levels were measured by the method of Omura and Sato ($\bar{x} \pm$ S.E., n = 5). Epoxide hydrolase activity was measured by incubating styrene oxide (1.6 mM) with 1.5 mg microsomal protein in a total volume of 400 µl for 15 min at 37° C. Unreacted styrene oxide was removed by petroleum ether extraction. Styrene glycol was subsequently extracted into ethyl acetate, the ethyl acetate layer was evaporated under nitrogen and the residue was reconstituted in 15% methanol/85% water for styrene glycol analysis by HPLC. Values for the nonenzymatic hydrolysis of styrene oxide were obtained from incubations using boiled microsomal enzymes; blank values have been substracted. Styrene glycol was quantitated by HPLC/UV 254 using a mobile phase of 85% water/15% methanol on a $C_{18}$ radial pak (5 x 100 mm) column at a flow rate of 1.0 ml/min. Two incubations were performed for each microsomal preparation, and values are means ± S.E. for the average styrene glycol formation in 5 preparations of microsomes.

glutathione (Figure 9). The rates of formation of conjugate 1 and 3 were similar in BHA and control microsomes while conjugate 2 formation was increased moderately (130% of control at 0.5 mM glutathione and 175% of control at 5 mM glutathione) in BHA compared to control microsomes (Figure 9). These data provide some support for the view that naphthalene epoxides may be the proximate metabolites involved in in vitro covalent binding of radioactivity from $^{14}$C-naphthalene (rather than metabolism to naphthol as proposed by Hesse and Metzger, 1979). The data showing that induction of epoxide hydrolase has relatively little effect on the rate of conjugate 1 or 3 formation and actually increases the rate of conjugate 2 formation indicates that the formation of dihydrodiol and glutathione conjugates are not competing pathways under these incubation conditions.

In comparison to the effects of induction of epoxide hydrolase (Figure 9), the data in Figure 10 indicate that inhibition of epoxide hydrolase by addition of cyclohexene oxide to the incubation results in no change in the rates of formation of metabolites which become bound covalently to microsomal protein in comparison to control. These findings support and extend the results of Hesse and Metzger (1979) using rat liver microsomes. Consistent with inhibition of microsomal epoxide hydrolase enzymes by cyclohexene oxide, there was a marked decrease in the formation of naphthalene dihydrodiol. Similar to the results of the experiment examining the effect of epoxide hydrolase induction, addition of cyclohexene oxide to the incubations resulted in no change in the rate of conjugate 1 or 3 formation and a significant increase in conjugate 2 formation. Although there is no obvious explanation for the data showing that induction or inhibition of epoxide hydrolase results in similar changes in the rates of conjugate formation, it is possible that the effect observed with BHA feeding is simply due to alterations in the P-450 isozymes that this agent may cause. Some evidence for this has been presented by Cha et al. (1979) who demonstrated that dietary BHA results in alterations of hepatic microsomal aminopyrene N-demethylase and aniline hydroxylase. Thus, while the experiments with epoxide hydrolase induction and inhibition are difficult to interpret completely, they do indicate that the ratio of cytochrome P-450 to epoxide hydrolase is not the primary determinant in the preferential formation of adduct 2 by lung microsomes. Furthermore, these studies indicated that with the incubation conditions used the dihydrodiol and conjugate pathways did not compete directly.

Comparison of Covalent Binding and Conjugate Formation in the Presence of Cytochrome P-450 Monooxygenase Inhibitors

Since monitoring the formation of glutathione adducts was initially intended as an alternative method for examining the rates of formation of individual reactive metabolites, it was important to determine whether there is a 1:1 relationship between these events (i.e. the same reactive metabolites which become bound covalently in incubations without glutathione form glutathione adducts in the presence of glutathione). As a means to test this, the effects of addition of SKF 525A or piperonyl butoxide on covalent binding and conjugate formation were determined in liver microsomal incubations conducted in the presence or absence of glutathione. Covalent binding of reactive naphthalene metabolites in incubations without glutathione was decreased markedly by addition of SKF 525A or piperonyl butoxide (Table 6, Figure 11). In contrast, addition of either of the monooxygenase inhibitors had only a moderate effect on the rate of conjugate formation but markedly decreased covalent binding in incubations containing glutathione. Covalent binding, therefore, appears to be considerably more sensitive to monooxygenase inhibition by SKF 525A and piperonyl butoxide, and thus the metabolites which become bound covalently may not be identical to those which form glutathione adducts. Additional studies are needed, however, to support this view. As stated by Gillette et al. (1982), a plot of covalent binding/conjugate formation vs [1/glutathione] should be linear if the same intermediate(s) is involved in covalent binding and conjugate

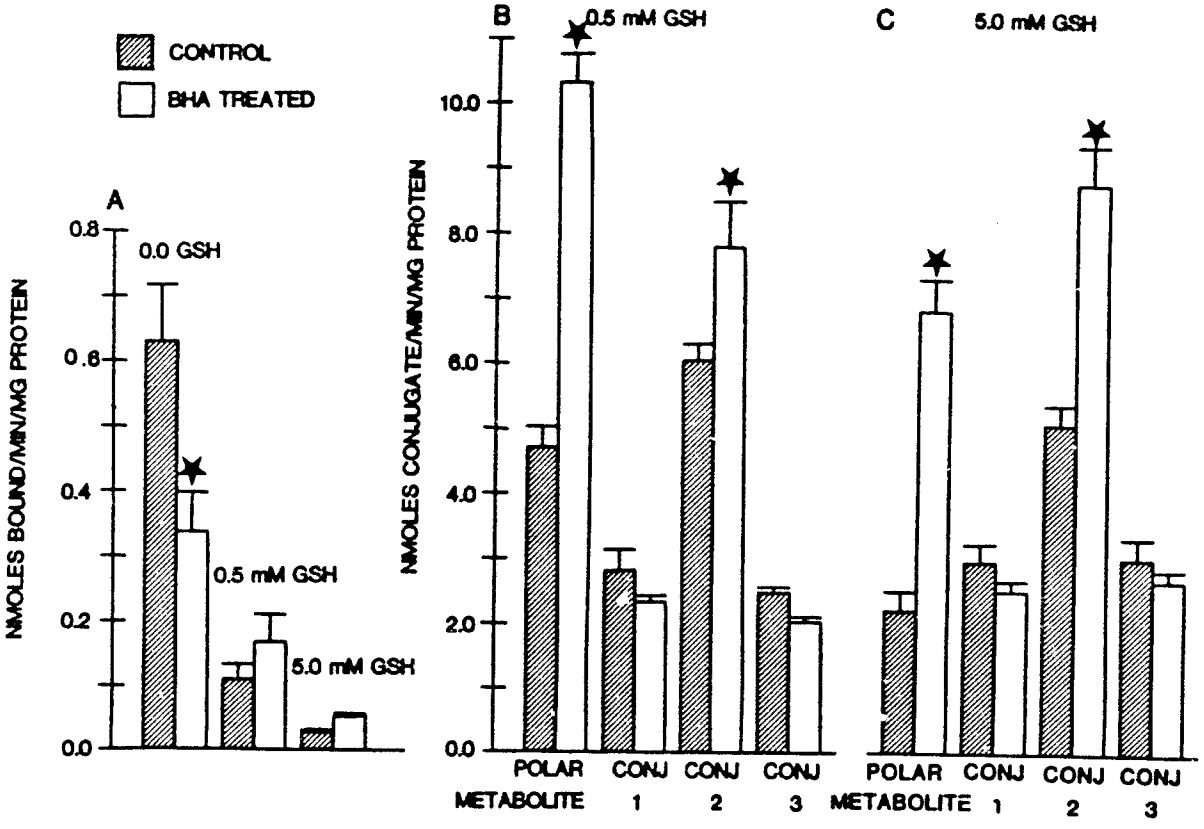


Figure 9. Effect of dietary BHA on liver microsome-catalyzed metabolism of naphthalene to covalently bound products and to glutathione adducts. Microsomes (1 mg), prepared separately from 5 control and 5 BHA treated mice, were incubated with 2 mg cytosolic enzymes (cytosol from control mice was used throughout), $^{14}$C-naphthalene (1 mM, 1168 dpm/nmole) and NADPH generating system and glutathione where indicated. Incubations were for 6 min at 37° C. Values are the means ± S.E. for 5 incubations.

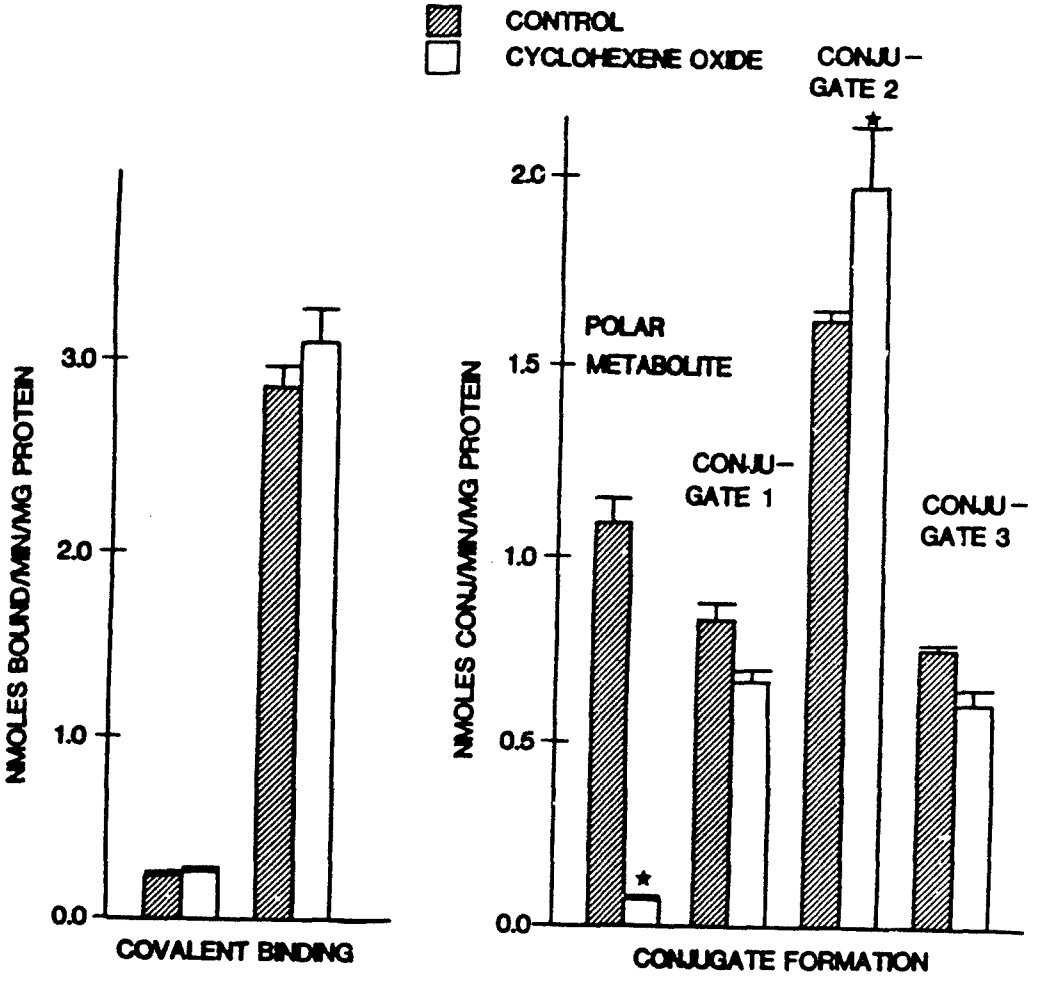


**Figure 10. Effect of addition of cyclohexene oxide on the covalent binding of reactive metabolites and on the formation of polar metabolite 1 and naphthalene glutathione adducts. In a total volume of 2 mg, each incubation vessel contained: 2 mg mouse liver microsomal protein, 2 mg cytosolic enzymes, 1 mM (1464 dpm/nmole) $^{14}$C-naphthalene, 5 mM glutathione (where indicated) and 0.5 mM cyclohexene oxide (dissolved in 10 µl acetonitrile, where indicated). Incubations were for 15 min at 37°C. Values are the mean ± S.E. for three incubations.**

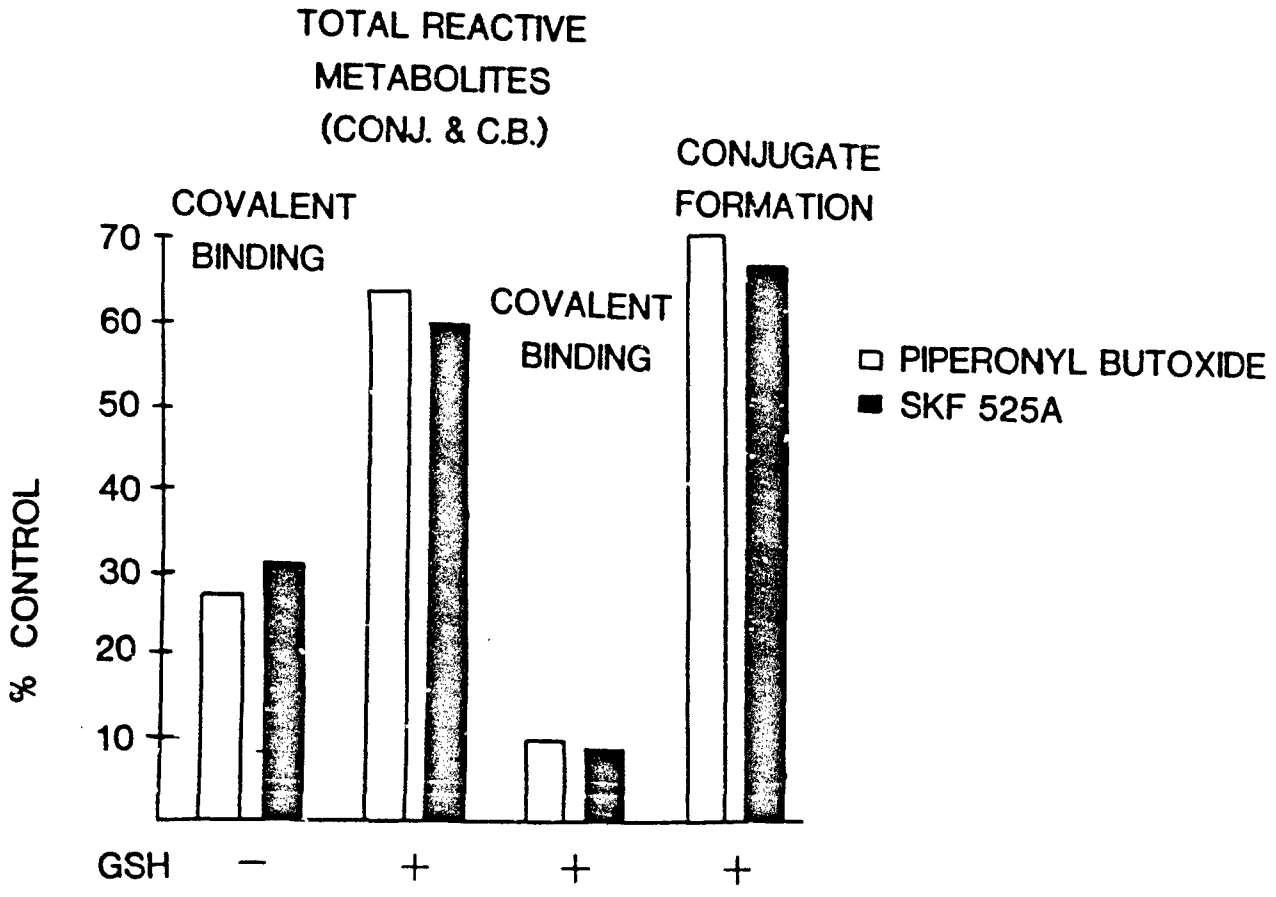


**Figure 11. Effect of addition of SKF 525A or piperonyl butoxide on the rate of reactive naphthalene metabolite formation as measured by covalent binding or the formation of glutathione conjugates. Data are from Table 6 and are expressed as a percentage of the no inhibitor control.**

Table 6

Effect of Varying Incubation Conditions on the Covalent Binding of Reactive Naphthalene Metabolites and on the Formation of Naphthalene Glutathione Adducts[a]

| Contents of Incubation | | | | | Polar Naphthalene Metabolite Formation nmoles/min/mg | | | |
|---|---|---|---|---|---|---|---|---|
| NADPH | GSH | SKF | PIP | Covalent Binding nmoles/mg/15 min | Dihydrodiol | Conj 1 | Conj 2 | Conj 3 |
| - | - | - | - | .017 ± .002 | - | - | - | - |
| + | - | - | - | 10.22 ± 0.26 | 1.95 ± 0.04 | 0.03 | 0.05 | 0.05 |
| + | - | + | - | 3.15 ± 0.11 | 2.59 ± 0.30 | 0.02 | 0.03 | 0.02 |
| + | - | - | + | 2.79 (n = 2) | 2.84 | 0.01 | 0.02 | 0.0[illegible] |
| + | + | - | - | 7.86 ± 0.34 | 1.52 ± 0.15 | 1.35 ± 0.15 | 2.08 ± 0.24 | 0.65 ± 0.07 |
| + | + | + | - | 0.73 ± 0.02 | 1.75 ± 0.02 | 0.61 ± 0.01 | 1.63 ± 0.04 | 0.50 ± 0.02 |
| + | + | - | + | 0.77 ± 0.07 | 2.99 ± 0.06 | 0.62 ± 0.04 | 1.83 ± 0.06 | 0.45 ± 0.02 |

[a]Incubations contained 4 mg phenobarbital-induced mouse liver microsomes, $^{14}$C-naphthalene (0.5 mM, 1242 dpm/nmole) and NADPH generating system, glutathione (1 mM), SKF 525A (0.5 mM) or piperonyl butoxide (0.5 mM) where indicated. Incubations were run at 37°C for 15 min. Values are the mean ± S.E. for 3 incubations.

formation. This experiment will be done later this contract year as part of the planned kinetic studies.

Addition of either SKF 525A or piperonyl butoxide to incubations done with or without glutathione increased the rate of formation of naphthalene dihydrodiol compared to control. This finding is difficult to reconcile with the data showing that the formation of metabolite 1 is NADPH-dependent. It is possible that, with the long incubation times used, microsomal cytochrome P-450 is quickly inactivated by the binding of reactive metabolites in incubations without inhibitor but that in incubations containing SKF 525A or piperonyl butoxide, the dihydrodiol is produced at a constant rate throughout the incubation period.

**Glutathione Adduct Formation as a Measure of Reactive Metabolites Formation from Naphthalene and 1-Naphthol**

Studies with benzene (Tunek et al., 1978, 1979; Sawahata and Neal, 1983), bromobenzene (Hesse et al., 1980), and naphthalene have suggested that the cytochrome P-450 mediated formation of covalently bound metabolites from these aromatic hydrocarbons is a two-step process involving the formation of a monohydroxylated derivative followed by the metabolic activation of phenol, p-bromophenol or 1-naphthol to intermediates which become bound covalently to tissue macromolecules. Tunek et al. (1978) and Sawahata and Neal (1983) have shown that the glutathione and N-acetyl cysteine adducts formed during microsomal incubations of benzene and phenol are identical. Moreover, the finding that benzene oxide does not become bound covalently to protein in vitro supports the view that intermediates from benzene which bind covalently are formed through phenol. Since the evidence for a similar mechanism with naphthalene is less convincing, a series of experiments were done in an effort to determine the contribution of intermediates from naphthalene and from 1-naphthol in in vivo and in vitro covalent binding.

To determine whether glutathione adducts formed microsomally from 1-naphthol were identical to those isolated from microsomal incubations of naphthalene, hepatic microsomes

prepared from phenobarbital-induced mice were incubated with NADPH generating system, cytosol, $^{14}$C-labelled substrate in the presence or absence of glutathione. As shown previously, addition of glutathione to incubations of microsomes, cofactor, cytosol and naphthalene decreased the covalent binding of reactive metabolites (Table 7). Likewise, the covalent binding levels in incubations containing 1-naphthol were decreased by addition of glutathione. High pressure liquid chromatography of an extract prepared from incubations of naphthalene, glutathione and NADPH yielded the dihydrodiol and three glutathione adducts as described previously. Chromatography of extracts prepared in an identical fashion from incubations containing 1-naphthol did not result in the elution of any radioactive or UV absorbing peaks in the region where the glutathione conjugates of naphthalene elute. Thus, naphthol is not an intermediate in the formation of any of the glutathione conjugates produced during the microsomal metabolism of naphthalene. A quantitative method for monitoring glutathione adducts from 1-naphthol is currently being developed so that the possibility of a heretofore unidentified naphthalene glutathione adduct which elutes with conjugates from 1-naphthol can be excluded.

Table 7

Effect of Addition of Reduced Glutathione on the Covalent Binding of Reactive Metabolites from Naphthalene and 1-Naphthol*

| Substrate | Glutathione | Covalent Binding nmoles/mg/min |
|---|---|---|
| Naphthalene | - | 10.2 ± 0.3 |
| | + | 7.8 ± 0.3 |
| 1-Naphthol | - | 13.0 (n = 2) |
| | + | 10.6 ± 0.5 |

*Incubations contained: 4 mg hepatic microsomal protein (from phenobarbital-induced mice), 2 mg cytosolic enzymes, $^{14}$C-naphthalene (0.5 mM, 1242 dpm/nmole) or $^{14}$C-1-naphthol (0.5 mM, 1047 dpm/nmole) and were done in the presence or absence of glutathione (1 mM) as indicated. Incubations were conducted for 15 min at 37° C.

Covalent Binding, Glutathione Depletion and Tissue Necrosis After Treatment with 1-Naphthol

If 1-naphthol is an obligate intermediate in the covalent binding, glutathione depletion or tissue damage by naphthalene, the administration of 1-naphthol should yield dose response curves for these phenomena which are shifted to the left. To examine this possibility, groups of 5 male Swiss Webster mice were treated with corn oil or 1-naphthol, intraperitoneally, at doses of 50, 100, 200 or 400 mg/kg. At 24 hours, surviving animals were given an overdose of pentobarbital, and lungs were fixed by tracheal infusion of Karnovsky's fixative. Tissues were embedded in paraffin, sectioned at 5-6 μ, stained with hemoxylin and eosin and examined by light microscopy. The data in Table 8 indicate that the acute toxicity of 1-naphthol is higher than naphthalene with 4 out of 5 mice dying within 2 hours of 1-naphthol (400 mg/kg) administration. Bronchiolar necrosis was not observed in animals treated with corn oil or 1-naphthol (50-400 mg/kg).

To determine whether 1-naphthol or its metabolites can become bound covalently to tissue macromolecules or can deplete tissue reduced glutathione, $^{14}$C-1-naphthol was administered to groups of 4 mice each at doses ranging from 25 to 200 mg/kg at the following

Table 8

Mortality and Tissue Necrosis 24 Hours
After 1-Naphthol

| Dose | Mortality % | Tissue Damage Lung |
|---|---|---|
| 0 | 0 | 0 |
| 50 | 0 | 0 |
| 100 | 0 | 0 |
| 200 | 0 | 0 |
| 400 | 80% | 0 |

specific activities (25 mg/kg - 1233 dpm/nmole; 50 mg/kg - 660 dpm/nmole; 100 mg/kg - 290 dpm/nmole; 200 mg/kg - 172 dpm/nmole). Covalent binding and reduced sulfhydryl levels were measured in tissues removed 2 hours later. The data in Figure 12 indicate that after the 50 and 100 mg/kg doses of 1-naphthol, glutathione levels were significantly higher than control. While sufficient information is not available from these studies to understand the underlying reason for this increase, it is possible that naphthol produces small decreases in pulmonary glutathione at early time points which stimulates subsequent synthesis. Similar phenomena have been noted previously in quail treated with 4-ipomeanol (Buckpitt et al., 1982). At all doses of 1-naphthol studied, covalent binding was low. Macromolecular covalent binding was dependent upon the dose and was highest in kidney followed by liver, lung and muscle. In comparison to these findings, covalent binding in the liver after a 400 mg/kg dose of naphthalene is approximately 1 nmole/mg protein. Thus, if treatment with 200 mg/kg 1-naphthol or 400 mg/kg naphthalene results in comparable tissue levels of 1-naphthol, the binding of reactive metabolites occurring after administration of naphthalene cannot be solely dependent upon the formation of 1-naphthol as an intermediate.

**Binding of Reactive Naphthalene Metabolites to DNA**

These studies are being conducted in collaboration with Dr. Ronald Shank, Principal Investigator, Comparative Biochemistry and Metabolism I: Carcinogenesis. Dr. Shank has had considerable experience in examining the covalent interactions of electrophilic drug intermediates with DNA.

In Vivo Experiments

A series of three experiments have been completed to determine whether radioactivity from $^{3}$H-naphthalene becomes bound covalently to highly purified DNA. The purpose of the first experiment was to determine whether any radioactivity was bound to liver DNA of phenobarbital-induced mice 2 hours following ip administration of 350 mg/kg $^{3}$H-naphthalene (6 mCi/mmole; 16.5 mCi/kg). DNA was isolated from livers of 5 animals (10.28 g) by the method of Swann and Magee (1963) and the $A_{260}/A_{280} = 2.01$. The DNA was hydrolyzed in mild acid (0.1 N HCl at 70° C for 30 min), the solution was neutralized by the dropwise addition of 1 N NaOH and 10 ml ACS was added. A sample containing 19.7 mg DNA had 654 cpm or 33 cpm/mg DNA. While these studies were not conclusive because the possibility of tritium exchange could not be excluded, they indicated that the research was worthwhile pursuing. The level of DNA binding was similar to that found in mouse forestomach (target organ for B(a)P carcinogenesis) after in vivo treatment with benzo(a)pyrene (Anderson et al., 1981) but is several fold lower than liver DNA after treatment with this polycyclic aromatic hydrocarbon.

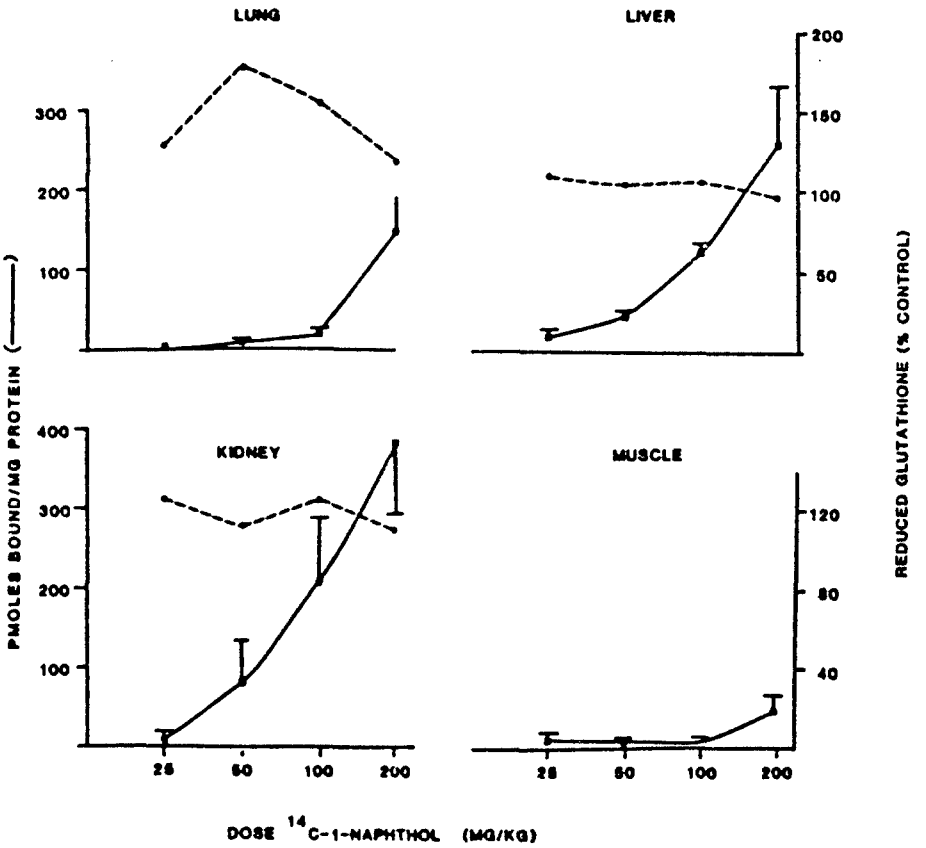


Figure 12. Covalent binding and tissue reduced glutathione levels in mice two hours after treatment with 1-naphthol ip. Values are mean ± S.E.

A total of 15 more mice were given phenobarbital (0.1%) in their drinking water for 5 days. $^{3}$H-Naphthalene (350 mg/kg, 16.5 mCi/kg) was administered ip to 10 animals while the remainder received corn oil alone. Animals were sacrificed 2 hours later and DNA was extracted as previously described. Thirty-five mg of DNA with an $A_{260}/A_{280}$ = 1.82 were isolated from livers of $^{3}$H-naphthalene treated animals. A 5 mg sample was removed and counted (32 cpm/mg DNA). The remainder was dissolved in 10 mM cacodylate buffer pH 7.0 and 7 substituted purines were selectively removed by neutral thermal hydrolysis as described by Becker et al. (1980). After hydrolysis, 0.11 x vol of 1 N HCl was added, and the precipitate was separated by centrifugation. The supernatant was evaporated to dryness in vacuo and the sample was redissolved in 1 ml distilled water. An aliquot of this (800 µl) was injected onto a Whatman Partisil SCX column (0.94 x 25 cm) eluted with water for the first 5 min followed by ammonium phosphate (70 mM, pH 2.0) at a flow rate of 5 ml/min. Column effluent was monitored with a Perkin Elmer fluorescence detector at λex 290 ± 12/λem 360 ± 12 and was collected at 30 sec intervals for the first 7 min and at 1 min intervals thereafter for scintillation counting. A fluorescence intensity and radioactive elution profile is shown in Figure 13. The major portion of radioactivity elutes in the column void volume. Two additional peaks elute after guanine at 12.5 and 16.5 min after injection. These data are consistent with the formation of a 7-substituted guanine or adenine adduct in vivo after naphthalene treatment.

The precipitated DNA from the neutral thermal hydrolysis was hydrolyzed in mild acid and was chromatographed on a strong cation exchange column under conditions identical to those used for the neutral thermal hydrolysate. Fractions of column eluate were collected at 1 min intervals for liquid scintillation counting. All of the radioactivity eluted near the column void volume in the pyrimidine oligonucleotide fraction.

To confirm these studies, a total of 30 mice were treated with phenobarbital in the drinking water for 5 days. Groups of 10 mice each were administered corn oil, 350 mg/kg $^{3}$H-naphthalene (15.7 mCi/kg, 12.7 dpm/pmole) or 35 mg/kg $^{3}$H-naphthalene (10.1 mCi/kg) 84.5 dpm/pmole) ip. A total of 24, 23 and 16.5 mg DNA were isolated from livers of control, 350 mg/kg and 35 mg/kg treated animals, respectively. The ratio of $A_{260}/A_{280}$ was 1.91 (control), 1.89 (350 mg/kg) and 1.86 (35 mg/kg). A 5 mg piece was removed from each sample, treated with DNase and counted for 50 min. The data in Table 9 show that approximately the

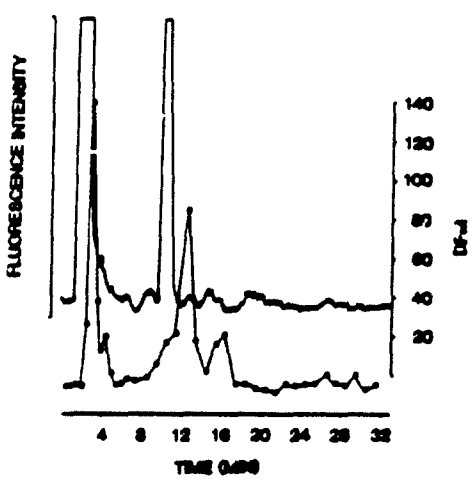


Figure 13. Fluorescence intensity and radioactive elution profile of the neutral thermal hydrolysate of 29.4 mg DNA isolated from the livers of mice treated with $^{3}$H-naphthalene (350 mg/kg) 2 hours earlier. The hydrolysate was chromatographed on a Whatman SCX (0.94 x 25 cm) column in water for 5 min followed by 70 mM ammonium phosphate buffer, pH 2.0 at 5 ml/min. Fluorescence intensity was monitoted with a Perkin Elmer 650-10S fluorimeter at 290 ± 12 nm (ex) and 360 ± 12 nm (em). Column eluate was collected and counted in a liquid scintillation counter for 50 min per sample. Efficiency was determined by internal standardization with $^{3}$H-toluene.

Table 9

Binding of Radioactivity from $^{3}$H-Naphthalene to Mouse Liver DNA In Vivo*

| Dose | Specific Activity of Dose Solution | cpm/mg DNA | pmoles/mg DNA |
|---|---|---|---|
| 35 | 84.5 dpm/pmole | 10.2 | 0.51 |
| 350 | 12.7 dpm/pmole | 37.4 | 12.5 |

*Groups of 10 mice each received $^{3}$H-naphthalene ip 2 hours before sacrifice. DNA was isolated from the livers by the phenol extraction method, and a 5 mg piece was removed, digested with DNase and counted for 50 min each. Efficiency was determined by internal standardization with $^{3}$H-toluene.

same level of binding was achieved at the 350 mg/kg dose as in past experiments, and that binding is more than 20 times greater at the 350 mg/kg dose than the 35 mg/kg dose. These data also indicate that the radioactivity associated with the DNA is unlikely to be due solely to $^{3}$H exchange since the amount of radioactivity administered to the 35 mg/kg treated group was approximately 60% of the 350 mg/kg treated group yet the specific activity of DNA of the high dose group was nearly 4 times that at the lower dose.

## SUMMARY AND CONCLUSIONS

Prior studies evaluating the relationships between the cytochrome P-450 dependent formation of reactive metabolites from naphthalene, and the highly cell, tissue and species selective pulmonary bronchiolar necrosis by this compound have demonstrated a close interrelationship between toxicity, glutathione depletion and covalent binding. For example, pretreatments with agents that alter the activity of the cytochrome P-450 monooxygenases or which alter detoxication pathways such as by glutathione, result in parallel changes in the severity of bronchiolar necrosis and the covalent binding of reactive metabolites in target tissue. However, the covalent binding of reactive naphthalene metabolites to macromolecules in nontarget tissues is always higher than it is in the lung. A possible explanation for this, which is still consistent with a role for the cytochrome P-450 dependent formation and covalent binding of reactive metabolites in naphthalene-induced pulmonary bronchiolar damage, is that several reactive metabolites capable of binding covalently but with different toxic potency are produced from naphthalene by lung and liver. Since glutathione had been shown to play a key role in modulating naphthalene-induced pulmonary lesions and since the formation of a glutathione adduct had been reported previously with naphthalene (Booth et al., 1961; Jerina et al., 1970), it seemed possible that reactive naphthalene metabolites could be trapped with glutathione and that analysis of the resulting thiol adducts could not only yield structural information as to the nature of the precursor reactive metabolites but could serve as a quantitative method for measuring specific reactive metabolites in vitro.

A new HPLC method was developed during this contract year which is capable of separating 1,2-dihydroxy-1,2-dihydronaphthalene and three naphthalene glutathione conjugates. The finding that multiple glutathione adducts are formed during the microsomal metabolism of naphthalene is consistent with other studies demonstrating the regio- and stereospecific formation of glutathione adducts from aromatic and aliphatic epoxides (Yagen et al., 1981; Van Bladeren et al., 1982).

The present studies support the view that the formation of a particular reactive metabolite (which results in conjugate 2) may be the primary determinant in the target tissue specificity by naphthalene. In all of the experiments reported here, the relative rate of formation of conjugate 2/1 and 2/3 was always greater in mouse lung microsomal incubations than in liver. Preliminary studies conducted with lung microsomes from the hamster (a species less sensitive to naphthalene-induced bronchiolar damage) indicate that the overall rate of conjugate formation was considerably less than in the mouse and that the profile of glutathione adducts formed is similar to the nontarget tissues of the mouse. However, there appears to be moderate interexperimental variability in the ratios of formation of the individual conjugates by lung microsomes. In some experiments the ratio of conjugates 2/1 and 2/3 was approximately 5/1 while in others the ratio was 15/1. While the reason for these differences is unclear, the variability does not appear to be associated with the analytical methodology used since the data obtained with separate microsomal incubations is highly consistent. Additional studies are being conducted currently in an effort to identify each of the glutathione adducts and these may shed some light on those factors likely to control the relative ratios of conjugates produced. Furthermore, before any definitive conclusions can be drawn as to the relevance of preferential formation of the intermediate which is conjugated to form adduct 2, studies on the relative conjugate ratios in lungs from sensitive and nonsensitive species and target and nontarget lung cells will be needed.

The finding that there is no detectable lag time in the formation of any of the conjugates and that relative rates of formation of each of the conjugates remain relatively stable with increasing microsomal protein concentrations and increasing incubation times, indicates that the formation of any one of the conjugates probably does not involve subsequent metabolism of one of the other conjugates. The possibility that conjugates are formed from intermediates which require recycling through the monooxyenase system cannot be excluded and must await structural identification of the adducts. However, preliminary studies indicate that none of the conjugates arise from metabolism of naphthalene to 1-naphthol.

The requirement for the cytosolic glutathione transferases confirms earlier studies on conjugate formation with naphthalene-1,2-oxide (Hayakawa et al., 1974, 1975) and suggests that, in vivo, the noncatalytic formation of thiol adducts from the precursor naphthalene intermediate(s) is negligible (see discussions by Ketterer et al., 1983). The present studies on naphthalene glutathione adduct formation add further support to the view that glutathione transferases play an important in vivo role in the conjugation of toxicants which show a glutathione dose threshold. Significant covalent binding of reactive metabolites and target tissue toxicity are not observed with toxicants like acetaminophen (Mitchell et al., 1973), bromobenzene (Jollow et al., 1974) and naphthalene (Warren et al., 1982) until doses sufficient to cause significant depletion of glutathione are achieved. In contrast, while glutathione appears to play an important role in modulating 4-ipomeanol-induced lung toxicity in the rat (Boyd et al., 1982) or hepatotoxicity in the bird (Buckpitt et al., 1982), covalent binding and target tissue toxicity do not appear to depend upon the substantial depletion of glutathione. In vitro studies have demonstrated that cytosolic glutathione transferases catalyze conjugate formation with microsomally derived reactive metabolites from bromobenzene (Monks et al., 1982) and naphthalene but had no effect on the formation of glutathione adducts with 4-ipomeanol (Buckpitt and Boyd, 1980).

Studies with partially purified glutathione transferases from livers of phenobarbital-induced mice indicated that under conditions where the metabolism of naphthalene to the intermediate(s) which conjugate with glutathione is rate limiting, the overall formation of naphthalene glutathione adduct 2 is more than three-fold higher than adduct 2 formation in the liver. Again these data support the view that the formation of a particular reactive metabolite

may be the primary determinant in the target tissue specificity by naphthalene. This contrasts significantly with the apparent mechanism of toxicity for 4-ipomeanol. This furan derivative elicits highly selective pulmonary toxicity in the rat which is paralleled by highly organospecific covalent binding of reactive metabolites in the lung. In vitro studies indicated that reactive metabolites produced by target and nontarget tissue microsomal incubations are identical and that these metabolites are produced in similar ratios by lung and liver (Buckpitt and Boyd, 1980). Thus, the organospecificity of damage by 4-ipomeanol appears to depend upon the kinetics of reactive metabolite formation rather than on the formation of a particular reactive metabolite as appears to be the case with naphthalene.

Alterations in the activity of microsomal epoxide hydrolase resulted in changes in the rates of dihydrodiol formation which were consistent with increases or decreases in the activity of this enzyme. Covalent binding in incubations of BHA microsomes done in the absence of glutathione was markedly decreased while, in contrast, inhibition of epoxide hydrolase by cyclohexene oxide had no effect on covalent binding. Although BHA feeding does not result in substantial alterations in overall cytochrome P-450 levels (Cha and Beuding, 1979; this study) it does change the rates of aminopyrene demethylation and aniline hydroxylation thereby raising the possibility that a decrease in covalent binding in BHA microsomes may be a result of altered activities of a P-450 isozyme responsible for metabolizing naphthalene to a covalently-bound product. The data showing an increase in dihyrodiol and conjugate 2 formation and no change in the rates of formation of conjugates 1 or 3 indicate that pathways for dihydrodiol and glutathione adduct formation may not compete effectively for the same intermediate. Further support for this possibility comes from the data showing that increasing the concentration of glutathione in the incubations from 0.5 mM to 5.0 mM markedly decreased the formation of the dihydrodiol yet had no effect on the rates of formation of any of the conjugates. In addition, overall conjugate formation was not affected by cyclohexene oxide even though dihydrodiol formation was decreased substantially.

The most direct evidence supporting the view that isozymes of cytochrome P-450 may control the relative ratios of glutathione adducts arising during the microsomal metabolism of naphthalene is that conjugate 2 formation was increased selectively in liver microsomes from 3-methylcholanthrene treated mice.

Pretreatment with both phenobarbital and 3-methylcholanthrene produced statistically significant increases in the rates of formation of all three glutathione adducts in lung microsomes. This contrasts with the data showing that these pretreatments decreased covalent binding of reactive naphthalene metabolites in lung microsomal incubation done without added glutathione and suggests that measurements of covalent binding and glutathione adduct formation may not yield equivalent results. Furthermore, studies in which the hepatic microsomal metabolism of naphthalene to covalently bound products and to glutathione adducts was inhibited by coincubation with piperonyl butoxide or SKF 525A showed that covalent binding is far more sensitive to inhibition by these cytochrome P-450 monooxygenase inhibitors than is conjugate formation. Interestingly, addition of either piperonyl butoxide or SKF 525A produced a more pronounced inhibition of the formation of conjugate 1 than of conjugates 2 or 3.

Lastly, additional evidence has been obtained in the studies conducted during the past year to indicate that reactive metabolites of naphthalene may become bound covalently to DNA in vivo, albeit at very low levels. These studies will require additional confirmation. DNA adducts formed in vitro during the microsomal metabolism of $^{3}$H-naphthalene are currently being analyzed in an effort to develop a specific and sensitive HPLC technique for monitoring the formation and fate of such adducts in vivo without the use (and attendant problems) of radiolabelled naphthalene.

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oU.S. GPO: 1983-659-005/210